Genetic Inheritance of Horticultural Traits in Faba Bean (*Vicia faba* L.)

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ABSTRACT

Faba bean (*Vicia faba*) has a relatively unexplored diversity in comparison to other legume crops. The large genome (13Gb), multiple centres of origin, and multiple domestication events resulted in vast diversity and many unexplored characteristics. Faba bean is both a grain legume and a vegetable, with its popularity ranging from being a common household food (Mediterranean and Asia) to being nearly unknown (North America). The horticulturally valuable traits of faba bean are very poorly researched. The main objective of this study was to determine the inheritance of the important characteristics for horticultural-type faba bean. Understanding these traits will aid in the expansion of faba bean into the horticulture market (ornamental and vegetable). Three traits were observed during this study to determine their inheritance: flower colour, plant height and branching. A selected diallel crossing block was performed and the subsequent F2 populations were phenotyped. The genetic sources for flower colour studies were P47-1 (red) and Gelber (yellow). Pearson chi-square goodness of fit tests were used to determine if the segregating F2 populations fit the hypotheses for a 15:1 ratio of red: non-red flower and 3:1 for brown: yellow wing spot. The chi- square value confirmed that red flower phenotype is controlled by two recessive genes and that the yellow wing spot is controlled by a single recessive gene ($p>0.05$). However, the inheritance of flower colour in faba bean is more complex than could be explained with these two hypotheses. To determine the inheritance of plant architectural characteristics, populations were developed using two different sources of dwarfism (NV153 and Rinrei) and one source of a highly branched phenotype (IG11476). F2 populations segregating for both sources of dwarfism were examined using a chi-square test. Populations segregating for both sources of dwarfism were tested for a single recessive gene model (3:1). Both dwarfs in this study fit this model based on visual characterisation, but only NV153 type dwarfs were true dwarfs. Rinrei type dwarf plants are more accurately described as slow growing plants. These plant types continued to grow throughout the season, resulting in non-significant height and internode data for dwarfism. The gene that causes the Rinrei type ‘slow growth’, does fit a 3:1 single recessive gene model and is pleotropic. The dwarf and slow growth gene are also additive and resulted in an extreme form of dwarfism at a ratio of 15:1. The highly branched trait did not fit into discrete categories and had a normal distribution characteristic of quantitatively inherited traits. The relationship between

flower colour and dwarfism with vicine-convicine was also analyzed by creating F2 populations that segregated for some of the horticulturally valuable traits and for the low vicine-convicine gene. Linkage was determined by chi-square analysis within each phenotypic class for the *–vc* gene that is known to be controlled by a single recessive gene and is not linked to red flower colour, yellow wing spot flower colour or Rinrei's 'slow growth' gene.

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DEDICATION

To my mother and father for their encouragement, support and confidence in me throughout my academic career.

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Comb.P	Combined P-value
Comb.X^2	Combined Chi-Square
d	Days
DF	Degrees of Freedom
F	Female
FIA	Flow Injection Analysis
FRET	Fluorescence Resonance Energy Transfer
G6PD	Glucose-6-Phosphate-Dehydrogenase
Gb	Gigabase
HCL	Hydrochloric acid
HET	Heterozygous
HOM	Homozygous
HVC	High Vicine Convicine
KASP	Kompetitive Allele Specific PCR
LVC	Low Vicine-Convicine
M	Male
Mt	Metric tonnes
Mbp	Megabase pairs
PCR	Polymerase Chain Reaction
SNP	Single Nucleotide Polymorphism
SRM	Selected Reaction Monitoring
VC	Vicine-Convicine

CHAPTER 1. INTRODUCTION

Faba bean (*Vicia faba* L.) is both a grain and a vegetable. In its horticultural form, pods are harvested when the seeds are filling, and the seeds are removed for fresh vegetable consumption. The horticultural type faba beans are consumed as a fresh vegetable in many countries around the world; however, this form of production in Canada is mostly limited to home vegetable gardens or small scale market gardening. In 2016, the horticulture industry in Canada had a farm-gate sales value of $2.3 billion (Agriculture and Agri-Food Canada, 2017). Horticultural products include vegetables, flowering plants, nursery stock, Christmas trees and turf sod. These industries are economically important, and therefore research into improved products can provide benefit to the horticulture sector.

Faba bean is an excellent source of dietary protein and is consumed in many countries around the world. It is a cool season legume with a large genome (13 GB) consisting of 12 chromosomes and has a vast amount of genetic diversity in traits including seed size, floral structure and both biotic and abiotic stress tolerance (Duc et al., 2010). Faba bean has a taproot system and fixes nitrogen in symbiosis with *Rhizobium leguminosarum*, bv. Viciae. Faba bean fixes 7-35% more nitrogen than any of the other major legume crops (Herridge et al., 2008). It is well adapted to temperate production zones in the northern hemisphere, Mediterranean winter/spring conditions and winter production zones of sub-tropical savannah regions (northern India as an example) with adequate seasonal moisture.

China is the largest producer of faba bean, producing 60% of the faba beans worldwide (Singh et al., 2013). However, no reliable data are available for fresh or frozen vegetable faba bean production (O'Sullivan and Angra, 2016). Faba bean production has been on the rise; in 2016, the production of faba bean was 4.6 Mt (FAOSTAT, 2018). The grain type faba bean for feed is small seeded (0.4-0.8 g dry matter/seed), often with reduced tannins (usually by incorporating the *zt1* gene) for improved nutrition and digestion when fed to monogastric animals. The vegetable type faba bean is large seeded (1-2 g dry matter/seed), and is often consumed fresh, canned or dried (Crepon et al., 2010). In Canada, the faba bean grain market is significantly smaller than that of other grain legumes, and the fresh vegetable faba bean market is minimal.

Although Canada has a substantial pulse industry (e.g., 2018, Canada produced 2.56 Mt of lentils, 4.11 Mt of dry peas, 0.10 Mt of chickpeas and 0.32 Mt of dry beans), the adoption of faba bean has been slow (Agriculture and Agri-Food Canada, 2019; Statistics Canada, 2015). Canadian

production of faba bean started in 1972; however, the amount of production has fluctuated (Oomah et al., 2011), and in 2017 it was 0.09 Mt (Agriculture and Agri-Food Canada, 2019). With the right advances in research and development, faba bean has enormous potential for growth in Western Canada based on the current expansion of the plant-based protein industry.

The floriculture industry is another unexplored market for faba bean. Faba bean has large variation for many ornamental traits such as flower colour, height and architecture. Aesthetic traits such as flower colour and plant architecture of faba bean were first reported by Erith (1930) to have qualitative (monogenic) inheritance, exhibiting classical conformation to the laws of (i) segregation and (ii) independent assortment of alleles. The first step to developing successful horticultural faba bean cultivars is an understanding of the genetics for the potential aesthetic and nutritional traits, especially flower colour, plant architecture and antinutritional factors. Faba beans have large clusters of fragrant flowers in an array of colours and patterns. By creating combinations of these unique characteristics, faba bean has the potential to enter the ornamental and floriculture markets. Faba beans have a semi-determinate growth habit and produce flower clusters from leaf axils. Variability exists for flower colour, size and number of flowers per cluster; however, flower colour is a primary floricultural breeding goal.

Many unique plant architectures exist within *Vicia faba* germplasm (dwarf, semi-dwarf, highly branched, etc.). The manipulation of these plant morphological characters can facilitate development of new products within the ornamental industry, since plant height and degree of branching are highly variable in faba bean. Faba bean plant height can vary from dwarf plants to towering giants. The semi-determinate nature of faba bean (Knott, 1990) and its large genetic variation results in enormous plants if grown in a long and favorable season, such as in New Zealand, where 2.5 m tall plants have been reported (Foundation for Arable Research, 2012). From a horticultural perspective, for example in greenhouse production, little research exists on how the size of a faba bean plant can be reduced. Branching pattern is also relatively unexplored from a horticultural perspective. Variation in branching was observed in a study of 13 genotypes where branch number per plant ranged from 2-11 (Chaieb et al., 2011). Plant height, node number and branching are important considerations for developing potential niche products within the horticultural market. A high degree of branching may be desirable for bedding and potted plants, however; the cut flower industry may require both long straight stems with no branching and highly branched plant types as is the case for many species used as cut flowers (e.g., carnations,

chrysanthemums, roses).

An antinutritional barrier to the successful integration and expansion of faba bean products into the Canadian food systems is the presence of vicine and convicine in seeds. A recessive gene for low vicine convicine (*vc*-) has been identified (Duc et al., 1989). The gene is located on chromosome I (Khazaei et al., 2015) and an effective SNP marker was recently developed (Khazaei et al., 2017) for use as a molecular marker. Since the location of flower colour and dwarfing gene(s) in faba bean are unknown, it is possible that they are linked with other known genes. By developing a segregating F2 population derived from crosses with a low vicine parent, linkage between low vicine and traits of interest can be determined.

This research has the primary goal of understanding the genetic inheritance governing the horticulturally valuable traits of faba bean. The motivation of this research is to facilitate the expansion of faba bean into the ornamental market.

The objectives of the studies were:

1) To determine the genetic control of the red and yellow spot flower colour
2) To determine the genetic control of two sources of dwarfism, prove independence and if they can be combined
3) To determine the inheritance of the highly branched trait
4) To determine the interaction between flower and stem traits with the low vicine-convicine gene (*vc*-).

1.1 Research Hypotheses

The research outlined below was designed to test the following hypotheses:

Hypothesis i: Red flower colour is a double recessive phenotype, controlled by two genes.

Hypothesis ii: Yellow wing spot is controlled by a single recessive gene.

Hypothesis iii: Dwarfism and semi-dwarfism (sourced from NV153 and Rinrei) in faba bean are controlled by two independent recessive genes that can be combined.

Hypothesis iv: The highly branched faba bean phenotype is controlled by a single dominant gene.

Hypothesis v: There is no linkage between the low vicine-convicine gene and any of the qualitative genes that result in the desired horticultural phenotypes in this research.

Note: Yellow spot was previously reported to be controlled by a single recessive gene (Cabrera 1988; Sjödin 1971). An unpublished undergraduate thesis at the University of Saskatchewan also reported red flower to be controlled by double recessive genes (Jans, 2013). This study will confirm and expand upon these reports.

1.2 Thesis Outline

The basic experimental approach was to develop genetic hypotheses for each objective and then design experiments by developing appropriate genetically segregating populations to test the hypotheses. Due to the multiple experiments conducted through this research, this thesis is organized into chapters by traits. All the chapters have the same initial population development, which was a diallel crossing block at the University of Saskatchewan (Chapter 3). To test the genetic hypotheses, the diallel crossing block was set up with 8 genetically distinct and homozygous parental lines, each with specific horticultural traits of interest. Targeted crosses were performed to develop genetic populations, and genetic analyses of the subsequent segregating populations were performed as described below. Within each chapter, the materials and methods used to further study the specific trait is outlined. Conclusions are drawn within each chapter, as in manuscript style, and a general discussion section ties everything together at the end.

CHAPTER 2. LITERATURE REVIEW

This review is focussed on the relevant phenotypic traits selected for study in the research objectives. The three themes in the review are the origins, the diversity and the genetics of horticultural traits of horticultural interest. Faba bean has an interesting origin, and many unanswered questions remain. The vast genetic diversity of *Vicia faba* has many horticulturally important traits which are examined throughout this review and study.

2.1 Faba Bean Origin and Diversity

Faba bean has a complex history. It is due to this complex history, extensive distribution, mating system and response to human selection that *Vicia faba* is one of the most variable species (Maalouf et al., 2013). The origin of domestication of *Vicia faba* was determined to be near Tell El-Kerkh in north-west Syria (Tanno and Wilcox, 2006) where archeological evidence of small-seeded faba beans were discovered that dated back to the 10^{th} millennium BC. The historical evidence of the large-seeded faba bean in the Mediterranean basin and Europe is much later, indicating it was a secondary centre of origin. From Europe, faba bean spread to South America with the Portuguese and Spanish explorers navigating the new world (Duc et al., 2010). As a result there are diverse landraces found in South America. Faba bean landraces are also found in China; however, the historical introduction is poorly understood. It seems likely faba bean was introduced to China with the development of the Silk Road. The domestication and cultivation of faba bean in these vastly different climates resulted in naturally selected genotypes suitable for the new environment. This extensive biodiversity enabled it to thrive in many locations and climates. Faba bean is primarily self-pollinating but outcrossing rates can vary from 20-80% depending on genotype and environment (Suso et al., 1999). The multiple climatically unique areas of domestication and the partial allogamous nature of faba bean facilitated the development of many diverse genotypes.

Faba bean phenotypes differ for numerous traits such as leaflet number, leaf colour, flower colour, dehiscence, stem height, degree of branching, flowering time, seed size, seed shape, seed colour, pod length and many others (De Ron, 2015). There are genotypes for different seasons, designated as spring-sown and autumn-sown types (Link et al., 2010). The different seasonal classes exhibit variation in vernalisation requirements, flowering times and

photoperiod response. This diversity was critical for the development of the distinct gene pools adapted to the different locations around the world.

Although there is a large amount of diversity, the wild progenitor of faba bean has either not been discovered, or is now extinct. The wild progenitor of faba bean is unknown, although Caracuta et al. (2016) discovered 14,000 year old specimens thought to be the lost progenitor of *Vicia faba*. In an attempt to identify the origin of *Vicia faba*, interspecific hybridizations were performed by Ramsay and Pickersgill in 1986 between *Vicia faba* (2n=12) and other species in the *Vicia narbonensis* complex (2n=14), with no success. *Vicia faba* has large portions of repetitive DNA causing it to have twice the amount of DNA compared to the other species in the *Narbonensis* complex (Duc, 1997). *Vicia faba* has the most nuclear DNA and largest chromosomes of the 56 *Vicia* species studied by Raina and Reese (1983). Although no progenitor species has been identified, the subspecies *Vicia faba paucijuga* is generally accepted as a primitive form of *Vicia faba* (Duc, 1997). There appears to be no wild faba beans since *Vicia faba* does not cross with other *Vicia* species; therefore, it is difficult to determine how much diversity has been lost since domestication. There are 37 *ex situ* faba bean collections around the world aimed at conserving the remaining diversity of the genetic resources for breeding and crop improvement (Duc et al., 2010).

2.2 Seed Components

Faba bean seed is diverse for size, shape and colour. The weight of faba bean seeds in the germplasm collections varies more than 10-fold (Maalouf et al., 2018), from 0.2 - 2.6 grams. Seed size is an important trait for large scale mechanized production, as large seeds are not compatible with standard seeding equipment. The shape of the seed also varies from large oblong and flat, to very small and spherical (Crépon et al., 2010). The colour of the seed coat can be yellow, green, black, brown, violet and many shades in-between, and the hilum can either be clear or black (Duc, 1997). As in most grain legumes, the seed contains proportions of starch, protein and fiber. Pulse crops are nutritionally important due to their relatively high amounts of protein (18-32%) (Boye et al., 2010). Although there is variability among genotypes, faba bean has a very high protein content, ranging from 24.7-37.2% (Duc et al., 1999; Duc, 1997; Crèpon et al., 2010). The other seed components of faba bean also vary depending on genotype: starch (24.7 - 37.2%), fibre (13.4 - 26.4%), sugar (3.5-6.3%) and crude fat (1.1-4.7%) (Duc et al.,

1999). The amino acid profile of faba bean is high in lysine, cysteine and tryptophan when compared to cereals (Duc et al., 1999) and high in lysine but low in cysteine and methionine when compared to soybean (*Glycine max*) (Duc, 1997). The storage proteins in faba bean seeds are globulins, albumins (water soluble) and glutelins (soluble in dilute acid and base) (Roy et al., 2010).

Faba beans also contain some anti-nutritional compounds: condensed tannins, vicine and convicine. Condensed tannins are proanthocyanidins which restrict the digestion of proteins in monogastric animals making them less desirable for the animal feed market. They also are associated with a bitter taste, making them undesirable in many crop species. Seed coat colour is an informative phenotype since tannin content impacts colour. The seed coat of low tannin faba beans are described as clear, beige, grey-beige, grey-green, grey or dark-grey. The seed coat of tannin-containing faba bean seeds are: black, brown, red, violet, green, yellow or beige. Seed coat colour remains stable in tannin-free seeds, however darkens over time in the tannin containing seeds (Crépon et al., 2010). The low tannin genes *zt1* and *zt2* have been identified (Picard, 1976) and result in seed coats that are tannin-free when homozygous recessive for either of these genes (Duc, 1997).

Vicine and convicine (VC) are primarily a concern in the human food market since they cause favism in humans with glucose-6-phosphate-dehydrogenase enzyme deficiency (Cappellini and Fiorelli, 2008). Favism results in hemolytic anemia in individuals with the enzyme deficiency after consuming faba beans containing VC. The majority of faba bean germplasm contains approximately 1% VC in the cotyledon tissue (Khazaei et al., 2017; Purves et al., 2017; Purves et al., 2018). VC concentrations are controlled by a single recessive gene (*vc*-). This gene reduces the VC 10-20 fold (Duc et al., 1989). The development of a KASP (Kompetitive Allele Specific PCR) marker has facilitated the rapid screening of low vicine-convicine (LVC) genotypes and improved breeding efficiency (Khazaei et al., 2017). Recently a unique and rapid flow injection analysis coupled with selective reaction monitoring (FIA-SRM) mass spectrometry method was developed to accurately detect LVC genotypes (Purves et al., 2018).

2.3 Plant Architecture and Impact

Plant architecture is “the set of features delineating the shape, size, geometry and external structure of a plant” (Ross, 1981). The size, shape and geometry of a plant is largely genetic;

however, plant architecture can also be influenced by environmental factors such as temperature, radiation intensity, day length, plant density and water availability (Huyghe, 1998). Plant architecture has a large impact on overall plant adaptation and reproductive success since it influences many of the critical processes for plant development. Architecture influences solar radiation absorption, transpiration rates, components of disease tolerance, water absorption and ultimately plant yield (Huyghe, 1998). The absorption of solar radiation in a plant depends on: leaf number, internode length, leaf size, leaf angle and canopy density (Sarlikioti et al., 2011). An experiment with tomato plants determined that long internode length and long narrow leaves led to more penetration of radiation throughout the plant canopy which increased yield (Sarlikioti et al., 2011). Leaf position was found to influence transpiration rates in Asian pear cultivars where leaves lower in the canopy had reduced transpiration (Xie and Luo, 2003). Leaflet number impacts disease tolerance by reducing the biomass loss when infection causes a leaflet to drop rather than an entire leaf, as seen in some *Cicer* species (Alberta Pulse Growers, 2019). A large plant is more competitive and therefore the leaf geometry affects the ability to outcompete neighbouring plants and impacts water absorption.

Faba bean exhibits a wide range of diversity of architectural characteristics. The stem traits vary for: height, internode length, degree of branching, growth habit and stem stiffness. This variation influence competitiveness in different climatic regions. The available growth habits and stem diversity of faba bean has facilitated breeding for different agronomic practices, such as intercropping (Duc et al., 2010). Faba bean root architecture is also a critical component of their success. Faba bean has a tap root with secondary roots that form nodules containing *Rhizobium leguminosarum*, bv. viciae. Through this symbiotic relationship, faba bean is able to fix atmospheric nitrogen into a plant available form. This deep tap root is a key feature of the species architecture by facilitating the production of nitrogen, and water absorption from deep in the soil profile.

By optimizing the architecture of a plant through breeding, the competitiveness can be enhanced. Faba bean grain producers in Western Canada desire tall, high yielding, and disease-tolerant varieties. Horticulturalists focussed on vegetable faba beans typically desire a synchronous high yield of pods. Seed catalogues of heirloom faba bean varieties often describe specific varieties in terms of flower size, fragrance, and growth habit. The vast diversity of plant structure enables development of multiple potential markets for horticultural faba beans.

2.4 Genomics and Genetics

Faba bean is a diploid species (2n=12) with a very large genome size. The genome of faba bean is 13 Gb., more than 25 times larger than the model legume *Medicago truncatula* which has a 470 Mbp genome (Young et al., 2003). The enormity of the faba bean genome is attributed to the large number of retrotransposon copies (Pearce et al., 1996) and repetitive DNA (Duc, 1997). The synteny between *V. faba* and *M. truncatula* resulted in the development of some successful primers in faba bean; however, relatively little is known of the genome of *Vicia faba* (Ray et al., 2015). In the 1960s and 1970s, faba bean was gaining popularity in cytogenetic models. However, the development of sequencing technologies shifted scientific research to new model organisms with small genomes, primarily *Arabidopsis thaliana*, and in the case of legumes, to *M. truncatula* and *Lotus* species. More recently, with the continual advancement of sequencing capability, a few grain legume genomes with smaller genomes are fully sequenced. At present, the effort to fully sequence the faba bean genome is in progress primarily through European research consortia. In general, faba bean genomic developments are more costly, and as a result, the available genomic tools for faba bean lag behind those available in many other legume crops (O'Sullivan and Angra, 2016).

Mendelian inheritance studies are useful for identifying the inheritance of qualitative traits. These have been used to describe many traits in faba bean such as hilum colour, seed coat colour, flower colour and dwarfism (O'Sullivan and Angra, 2016). In 1930 a study used Mendelian segregation ratios to determine that the normal flower colour (white with a brown wing spot) was dominant over white flowers (Erith, 1930). This study also identified one source of dwarfism (Little Marvel) and proved its inheritance to be determined by a single factor where tall is dominant. Additionally, the expression of a red-stemmed faba bean seedling was found to be controlled by a single dominant gene by calculating the goodness of fit test for the 3:1 Mendelian segregation ratio (Metz et al., 1992). Classic Mendelian genetic studies are an excellent tool for identifying inheritance of single genes; however, when a trait is controlled quantitatively other tools must be used. Quantitative traits are controlled by multiple genes, each with an effect on the expression of a specific trait. Yield is the classical and excellent example of a quantitative trait. Many genes will impact the yield of a plant, and quantitative yield data often are described as a normally distributed curve on a histogram as opposed to the bimodal nature of a classical qualitative trait.

2.4.1 Flower Colour & Wing Spot

Gregor Mendel's experiments on gene segregation are still relevant today. There are many examples of plants that follow Mendelian inheritance patterns for flower colour. Aside from pea, Mendel's model plant (Dunn, 2003), the flower colours of many other ornamental plants (for example, *Petunia hybrida* and *Stokesia laevis)* are controlled by qualitative genes that follow simple Mendelian genetics. The inheritance of anthocyanin in petunia flower is controlled by two independent genes (Griesbach, 2010). Stokes aster flower colour is controlled by at least three loci and is consistent with simple Mendelian genetics (Gaus et al., 2003). Due to these consistencies over many species, the hypothesis for flower colour in faba bean was developed based on simple Mendelian genetic models.

Faba bean flowers are typically papilionaceous (Duc, 1997), with five petals comprised of fused standard, wings and a fused keel petal. The wild type flower colour of *Vicia faba* is white with a dark spot on the wing petal and brown or violet stripes running vertically along the standard (Sjödin, 1971). Faba bean flowers are complete, containing both male and female reproductive organs, and flowers can therefore be both self-pollinated and outcrossed. The known colours of faba bean flowers are: white, white with brown wing spots, white with yellow wing spots, solid brown, solid brown and pink, diffused yellow and red (Cabrrera, 1988; Erith, 1930; Sjödin, 1971).

For this experiment, four different flower colours were included: red/purple, yellow spot, brown spot and white (*zt1* & *zt2* genes). In this study, Aurora was the source of wild type flower colour. In 1930 the first study of flower colour examined the inheritance of white flower, where it was determined to be a single recessive gene (Erith, 1930). Later in the 1900s, two independent white flower colour genes were identified (Picard, 1976). Both *zt1* and *zt2* genes are single recessive genes that result in zero tannin white flowers (Duc, 1997). These genes have pleotropic effects causing non-expression of the stipule spot and stem colour (Metz et al., 1992). The source of *zt2* in this study was Disco/2. Disco/2 is a Fevita® type faba bean, which means it is low tannin and low in VC (Duc et al., 2004). CDC Snowdrop is the source of *zt1* but it is not low in VC. The source of red flower colour comes from P47-1 and was thought to be controlled by a double recessive gene according to Brett Jans' undergraduate thesis in 2013 at the University of Saskatchewan. P47-1 was originally derived from crossing with the vegetable faba bean "1778" which was listed in a horticultural seed catalogue. (Note: many garden seed

catalogues offer this type under various names). The source of yellow spot flower came from a spontaneous mutation (Sjödin, 1971).

In 1971, Jan Sjödin identified four loci that affect flower colour and denoted them: *sp-a, sp-b, dp-a* and *dp-b*. He also determined that *sp-a* and *sp-b* are functionally independent loci causing the white flower colour phenotype; the genes are now known as *zt1* and *zt2* (Duc, 1997). Solid flower colour appears when the loci *dp-a* is homozygous recessive and the yellow wing spot appears when *dp-b* is homozygous recessive (Sjödin, 1971). The monogenic inheritance of the solid wing colour was corroborated by Cabrera (1988) and then determined to be segregating independently of the standard petal. Wing spot does not appear on the solid coloured flower mutants (*dp-a* locus), likely due to the visibility of the spot being masked by the solid flower colour. There has been limited research on the yellow spotted mutant. However, both Sjödin (1971) and Cabrera (1988) performed a cross between a yellow spotted type and a wild type plant which indicated monofactorial inheritance. Sjödin (1971) also found that all the flower colours were recessive to the wild type flower colour. Many shades of brown and pink flowers have also been recorded, but there is limited understanding on the inheritance of these colours.

Flower colour can also be used as a morphological marker indicating the presence or absence of condensed tannins. The wing spot on faba bean flowers has been observed in two colours, black/brown and yellow. There are also certain genotypes that lack the spot altogether. The dark spot indicates the presence of condensed tannins and anthocyanin in the flower and seed. Martin et al. performed an experiment in 1991 comparing tannin content in the seed with that in flower tissue and found that brown flowers had the highest mean tannin content when compared with white, yellow-spot, normal, diffuse yellow and diffuse brown. They also found that coloured flowers had higher overall tannins than spotted flowers. Although tannins are anti-nutritional, they often play an important role in plant defense mechanisms (Martin et al., 1991).

2.4.2 Dwarfism

Dwarf mutations exist in many of the major legume species, including faba bean (Huyghe, 1998). A study comparing plant height over 5000 faba bean accessions found the average height to be 78 cm, with a range of 10.3 - 201.5 cm (Zong et al., 2006). There are multiple sources of dwarfism in *Vicia faba*, both spontaneous and induced. Four sources of dwarfism have been reported in *Vicia faba* var. *minor* and two sources have been reported in

Vicia faba var. *major*. However, crosses between them have not been examined. The first four dwarfism genes recorded in *Vicia faba* var. *minor* are: *dw1, dw2, dw3* and *dw4*. The first three (*dw1, dw2* and *dw3)* are all spontaneous mutations; however, *dw4* was created through mutagenesis (Huyghe, 1998). The first published record of dwarfism in *Vicia faba* was *dw1* in 1962 (Bond and Fyfe, 1962). This dwarfing gene shows a significant reduction in height and flowers at 5-10 cm tall, but plants do not set seed and these plants need to be maintained in the heterozygous state. Nearly a decade later the second record of dwarfism was *dw2* was documented in Sweden by Sjödin (1971). The last type of spontaneous mutation (*dw3*) was found in the line HG115C during an analysis of variation study at ICARDA. The fourth dwarfing (*dw4*) gene was created by mutagenesis and was identified in the cultivar Fribo (Chapman, 1986). Sjödin reported that the dwarfism was caused by a reduction of internode length in the mutant. *Vicia faba* var. *major* also has two reported dwarf types, both created by mutagenesis. The first type of dwarfism was created by ethyl methane sulfonate, but no gene name was designated (Filipetti, 1988). The other type of semi-dwarfism was created through γ-ray irradiation, and was designated *dwarf-1* (Fukuta et al., 2004). Both dwarf mutations in *Vicia faba* var. *major* have pleotropic effects on the plant, resulting in a dark green foliage (Filipetti, 1988; Fukuta et al., 2004). No analysis comparing these two sources of dwarfism and their mechanisms has been reported.

There have been two mechanisms of dwarfism identified in faba bean, both the result of an incomplete hormone biosynthesis, either gibberellic acid or brassinosteroid. The mechanisms for *dw1* and *dwarf-1* are respectively known to be gibberellic acid deficient and brassinosteroid deficient mutants (Chapman, 1986; Fukuta et al., 2004). Gibberellins promote cell elongation, germination and flowering (Ogas, 1998). Gibberellin insensitivity is caused by mutations in the genes that encode gibberellin signaling proteins (Peng et al., 1999). This results in an abnormal response to gibberellins and reduced plant growth. Brassinosteroids are steroidal growth promoting plant hormones (Bajguz and Tretyn, 2003) that also serve as stress response hormones that protect plants from drought, salinity, heavy metals and herbicides (Sasse, 1999). Hormone biosynthesis is a multifaceted process, and disruption early in the pathway can lead to significant deficiencies in the plant. Some dwarf mutant plants can be restored to regular height through exogenously applied hormones, depending on whether the dwarfism is caused by a blockage in a pathway or a signaling mutation.

Both known dwarf-inducing deficiencies are examined in this study. One source is Rinrei, where the semi-dwarfism is caused by the *dwarf-1* gene. Rinrei is characterized by short internode length and short petiole length (Fukuta et al., 2004). Rinrei was created by gamma-ray irradiation that caused the plant to be defective in its ability to reduce sterol C-24, resulting in a brassinosteroid deficiency and therefore shortened stature (Fujioka et al., 2004). Rinrei also has other characteristic phenotypes such as lanceolate leaf shape and shiny dark green colour. The inheritance pattern of the trait is consistent with simple Mendelian inheritance for a single recessive gene. Expression of dwarfism in this case has pleiotropic effects on the plant; all plants expressing dwarfism inherited from Rinrei also expressed the unique foliage (Fukuta et al., 2004). The second source of dwarfism used in this experiment is NV153, a dwarf line only recently developed in the United Kingdom. It is considerably shorter than Rinrei, with prolific branching. The gene controlling NV153 has not be designated yet. However, the method of dwarfism is thought to be due to a block in the gibberellin pathways (Donal O'Sullivan, personal communication, 2017).

2.4.3 Branching

IG 114476 is a highly branched phenotype originating from Bangladesh. Understanding branching morphology is important in both large scale agronomic systems and large or small scale horticultural operations. The degree of branching varies in *Vicia faba*. In a collection of 5000 accessions, the number of branches ranged from 1.1 to 11.4 (Zong et al., 2006). The variation in branching has a climatic association whereby autumn-sown genotypes are known to have a higher degree of branching than spring-sown type faba bean, 4-6 and 1-2 branches, respectively (Duc, 1997). Autumn-sown genotypes are more likely to have basal branching than spring-sown genotypes. In temperate climates with short growing seasons, a high degree of late season branching can be detrimental to yield, since the plant's energy is being divided between setting seed and producing new branches (Huyghe, 1998). However, increased early branching and ground coverage provides improved weed suppression, and might have a positive effect on yield.

2.4.4 Vicine-Convicine

Vicine and convicine (VC) are glucosidic aminopyrimidine derivatives found in *Vicia* species (Pitz and Sosulski, 1979). Vicine [2,6-diamino-4,5-dihydroxypyrimidine 5-(β-D-glucopyranoside)] and convicine [2,4,5-trihydroxy-6-aminopyrimidine 5-(β-D-glucopyranoside)] are always found together and therefore are likely synthesized by the same pathway (Khazaei et al., 2015). Vicine and convicine have harmful effects on individuals with a glucose-6-phosphate dehydrogenase (G6PD) deficiency. This deficiency is a relatively common human enzyme defect (Cappellini and Fiorelli, 2008); therefore, VC are major concerns for the faba bean industry. Favism occurs in individuals with the X-linked mutant allele of the G6PD gene, after the consumption of faba beans. This clinical disorder appears due to the aglycone derivatives (divicine and isouramil) produced in the large intestine and caecum from the VC in faba beans (Jamalian and Ghorbani, 2005). This causes anemia in the individual, which in the most severe cases could be fatal. There are approximately 400 million individuals with favism around the world (Crépon et al., 2010). The frequency of this deficiency is much greater in malaria-prone countries, and it is thought to have increased in frequency in some human populations as a form of malaria resistance (Cappellini and Fiorelli, 2008).

Vicine and convicine can be toxic to individuals with a genetic deficiency of G6PD (Crépon et al., 2010). Since low VC is an important trait in faba beans for human consumption, it is of interest from both an agronomic and horticultural perspective. In this experiment, a parental line with the low VC gene (*vc*-) was crossed with the other parental lines to determine if linkages occur between *vc*- and the main horticulturally important traits.

The development of a high-throughput KASP marker has streamlined breeding for VC reduction. This KASP_*vcp2* marker is able to distinguish the *vc*- gene in both homozygous and heterozygous states from seed (Khazaei et al., 2017). This marker is an improvement from the previously reported flanking SNP marker. The previous marker was 1 cM and 2.6 cM away from the gene on either side (Khazaei et al., 2015).

2.5 Horticulture Industry Impact

Within the horticulture market there are many different sectors based on the product type. The three main areas that could be enhanced by developing new product through this research are (i) floriculture, (ii) nursery plants and (iii) garden bean vegetables. In 2017 these sectors were

worth $278 300 000, $456 000 000 and $36 127 000 respectively in Canada (Agriculture and Agri-Food Canada, 2017). Due to the incredible diversity for flower colour and plant architecture observed in this study, faba bean is a suitable species for developing new plant types to expand the horticulture industry in both in Canada and Internationally. Currently the other main legume used for an ornamental purpose is lupin (*Lupinus* spp.). Lupin, however; is less palatable for human consumption than faba bean. Additionally, lupin does not produce nectar and therefore does not provide an energy source for the visiting pollinators, which can reduce their longevity (Rasheed and Harder, 1997). In contrast faba bean does produce nectar and is therefore a pollinator friendly species.

CHAPTER 3. GENERAL MATERIALS AND METHODS

3.1 Parent Phenotype Selection

To capture the diversity of this study's traits of interest, a diverse set of parental lines were selected based on their horticultural phenotypes. Four of the parental lines were chosen based on their flower colour (Figure 3.1), two dwarf types, one highly branched type and one representative wild type (Table 3.1). In January 2017, four replications of the 8 parents in this crossing block were seeded at two week intervals (January 30th, February 13th, February 28th, and March 13$^{th)}$ to account for the variation in flowering time of the parental lines. Seeds were scarified and inoculated with *Rhizobium leguminosarum* bv. *viciae*. These seeds were planted into 3-gallon pots of soilless growing mixture No. 3 (Sun-Gro Horticulture MA, USA) and placed in a phytotron growth chamber in the Agriculture and Bioresources building at the University of Saskatchewan. The light condition in the phytotron chamber were 16 hours at a photon flux of 300 μmol $m^{-2}s^{-1}$ followed by 8 hours of dark. The temperature was 21 °C during the light interval, and dropped to 18 °C for the dark interval. Plants were watered as necessary and fertilized with a blend of 15-30-15(N-P-K) biweekly after the 10th node stage.

3.2 Insect control

3.2.1 Phytotron insect control

The main insect problem in the phytotron was thrips (*Frankliniella occidentalis).* Thrips consume the new shoot growth, and nectar and pollen of faba bean flowers. They are extremely prolific and therefore can be highly damaging. There are two methods of control for thrips in confined spaces: chemical or biological. They are difficult to chemically control since the insects are small and can avoid contact with chemicals by hiding in flowers and small folds of plant tissue. Therefore, biological control was used in the form of beneficial mites (*Amblyseius cucumeris*) and nematodes (*Steinernema feltiae*) to suppress the thrip population. The mites (250 000) were applied weekly directly to the leaves and growth points of the plants. Nematodes were applied in solution with water by spraying them onto the leaves and soil, in dark conditions biweekly. In 2018, pirate bugs (*Orius insidiosus)* were introduced biweekly instead of the nematodes. The pirate bugs were placed directly onto the leaves of the faba beans.

3.2.2 Field Insect Control

The early season pests that impacted faba bean growth were cutworms (*Agrotis orthogonia)*, and the mid-season pests were blister beetles (*Lytta nuttalli* and *Epicauta pennsylvanica)*. Both were controlled chemically by applying the contact insecticide Madator ™ at a rate of 34 mL/acre.

3.3 DNA Extraction for Sequencing

When the plants were 4 weeks old, DNA was extracted from all 8 parent genotypes to determine homozygosity and heterozygosity (Table 3.2), to ensure the crossing block was starting with genetically pure lines. The DNA extraction methods followed the procedures of the LGC groups (LGC Genomics, Beverly MA, USA) plant sample collection kit (Appendix C). Five leaf discs were harvested from the newest leaves of the plant and placed in a test tube. Between each plant sample extraction, the disc cutting tool was cleaned by depressing it 5-10 times in water and then drying it. The test tubes were placed in a 96-well tube rack, labeled and sealed. A desiccant sachet was placed on the 96-well tube storage rack, before bagging and preparing for shipment to the LGC lab. The LGC lab ran 875 SNPs (Webb et al. 2016; Cruz-Izquierdo et al. 2012; Ocaña-Moral et al. 2017) and purity was determined by calculating the percent homozygosity.

$$\frac{Homozygous\ SNP}{Total\ SNP}\ x\ 100 \tag{3.1}$$

Table 3.1: The characteristics of the 8 faba bean parent lines used to examine the horticultural traits of interest in this study

Plant Line	Standard Colour	Wing Colour	Wing Spot	Stipule Spot	Height	Branching	Tannins	HVC/LVC	Seed coat	Hilum	Origin/Source
Aurora	White	White	Brown	Present	Normal	Normal	Present	HVC	Beige	Black	Sweden
CDC Snowdrop	White	White	Absent	Absent	Normal	Normal	Absent	HVC	Light Beige	Brown	CDC, Canada
Disco/2	White	White	Absent	Absent	Normal	Normal	Absent	LVC	Grey/Beige	Colourless	INRA, France
Gelber	White	White	Yellow	Present	Normal	Normal	Present	HVC	Beige	Colourless	Svalöf, Sweden
P47-1	Red	Red	Brown	Present	Normal	Normal	Present	HVC	Green	Colourless	CDC, Canada
IG 114476	White	White	Brown	Present	Semi-Dwarf	High	Present	HVC	Black	Black	Bangladesh
Rinrei	White	White	Brown	Present	Semi-Dwarf	Normal	Present	HVC	Green	Black	Japan
NV153	White	White	Brown	Present	Dwarf	Normal	Present	HVC	Beige	Black	NIAB, UK

HVC=High vicine convicine
LVC=Low vicine convicine
NIAB=National Institute of Agricultural Botany
INRA=Institut National de la Recherche Agronomique

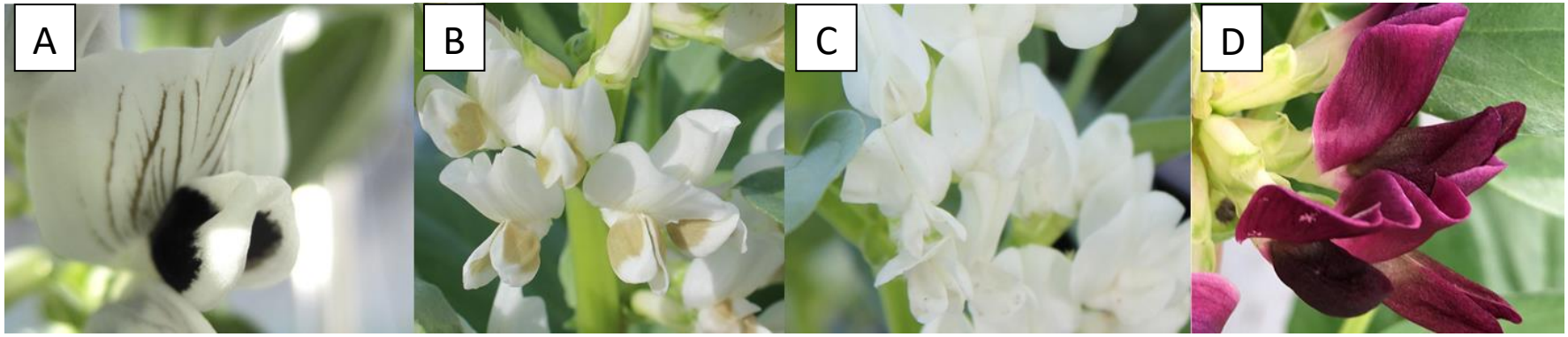

Figure 3.1: The four flower colours of the parent lines in the crossing block. A= wild type, B= yellow spot, C=white and D=red

Table 3.2: Homozygosity and heterozygosity percentage for 875 single nucleotide polymorphisms from 8 faba bean plant lines

Plant Line	% Hom	% Het
Aurora	92.85	7.20
CDC Snowdrop	95.00	4.99
Disco/2	98.96	1.04
Gelber	98.48	1.52
P47-1	98.95	1.06
IG114476	99.41	0.60
Rinrei	99.18	0.82
NV153-1	99.53	0.47

3.4 Crossing Procedure

To perform the selected crosses, young flowers were emasculated with sterilized tweezers (98% ethanol) before any of the anthers had reached anthesis. Next, a pollen donor flower from the male in the desired cross was selected. Pollen was harvested with sterilized (98% ethanol) tweezers by creating a vertical cut through the fused keel and collecting the loose pollen. The pollen was then transferred to the stigma of the emasculated female flower. The female peduncle was tagged, denoting the name of the male and female parents and the date. The goal for each cross combination was to obtain a population size that would give 99% probability level according to Muller's formula (Mainland, 1951) for the hypothesized number of genes (Table 3.3). Out of a total 334 crosses performed, 28% successfully yielded viable seed.

Muller's formula: $n = k\,(r + 1/2)$

(Mainland, 1951)

(3.2)

n = number of individuals

$k = -\log_e(1 - p)$

r = a/b, the ratio between expected number of non-desired types (a) over the expected number of desired types (b).

p=desired probability level

Table 3.3: Minimum population sizes to determine three genetic segregation ratios at two probability levels according to Muller's formula

Mendelian F2 Ratio	Probability	
	95%	99%
3:1	10.4	16.0
15:1	46.4	71.4
63:1	190.2	292.4

(Mainland, 1951)

3.5 Genetic Analyses

Selected crosses were made between parents in the crossing block (Table 3.4) to obtain F1 seeds which were harvested and planted on June 24th of 2017. Each F1 seed was scarified and inoculated before being planted in 2 or 3 gallon pots. Each pot was labelled with a designated plant number and pedigree, and were grown in the phytotron chamber at the University of Saskatchewan under the same conditions as previously described (Section 3.1). Some of the F1 flowers were backcrossed to their respective parents to perform test crosses. The remaining F1 flowers were allowed to self-pollinated to create the F2 generation. Two sets of F2 plants were grown, the first in the phytotron at the University of Saskatchewan and the second in the field at the Crop Science Field Laboratory at University of Saskatchewan. Each F2 plant was grown and data were recorded for plant height, flower colour and branching characteristics.

Table 3.4: Diallel crossing block for 8 faba bean plant lines. Selected crosses were performed to test the inheritance of the traits of interest in this study

F\M	Rinrei	NV153	IG 114476	P47-1	CDC Snowdrop	Disco/2	Aurora	Gelber
Rinrei	x	Rinrei X NV153	Rinrei X IG 114476	Rinrei X P47-1	Rinrei X CDC Snowdrop	Rinrei X Disco/2	Rinrei X Aurora	Rinrei X Gelber
NV153	NV153 x Rinrei	x	NV153 X IG 114476	NV153 X P47-1	NV153 X CDC Snowdrop	NV153 X Disco/2	NV153 X Aurora	NV153 X Gelber
IG 114476	IG 114476 X Rinrei	IG 114476 X NV153	x	IG 114476 X P47-1	IG 114476 X CDC Snowdrop	IG 114476 X Disco/2	IG 114476 X Aurora	IG 114476 X Gelber
P47-1	P47-1 X Rinrei	P47-1 X NV153	P47-1 X IG 114476	x	P47-1 X CDC Snowdrop	P47-1 X Disco/2	P47-1 X Aurora	P47-1 X Gelber
CDC Snowdrop	CDC Snowdrop X Rinrei	CDC Snowdrop X NV153	CDC Snowdrop X IG 114476	CDC Snowdrop X P47-1	x	CDC SnowdropX Disco/2	CDC Snowdrop X Aurora	CDC Snowdrop X Gelber
Disco/2	Disco/2 X Rinrei	Disco/2 X NV153	Disco/2 X IG 114476	Disco/2 X P47-1	Disco/2 X CDC Snowdrop	x	Disco/2 X Aurora	Disco/2 X Gelber
Aurora	Aurora X Rinrei	Aurora X NV153	Aurora X IG 114476	Aurora X P47-1	Aurora X CDC Snowdrop	Aurora X Disco/2	x	Aurora X Gelber
Gelber	Gelber X Rinrei	Gelber X NV153	Gelber X IG 114476	Gelber X P47-1	Gelber X CDC Snowdrop	Gelber X Disco/2	Gelber X Aurora	x

CHAPTER 4. FLOWER COLOUR GENETICS

4.1 Abstract

The available information on the genetics of flower colours in *Vicia faba* L. is limited. This lack of understanding has reduced the inclusion of unique flower colours into the vegetable type faba bean market. The inheritance of two flower colours (red corolla and yellow spot on wing petals) were examined through the development of multiple F2 and F2:3 segregating populations. The inheritance of red flower was confirmed for two recessive genes ($p>0.05$) and yellow wing spot inheritance was confirmed for a single recessive gene ($p>0.05$). These populations led to the discovery of a red and yellow flower colour, which has never been previously reported. Flower pattern genetics are also important for the development of new varieties. The solid wing colour gene was confirmed as a single recessive gene. Understanding the inheritance of flower colour in faba bean improves current vegetable types and opens up the possibility for ornamental markets.

4.2 Introduction

Flower colour in faba bean is an important trait for the expansion of faba bean into the horticultural market. Limited flower colour diversity is available in the current grain type varieties since they are grown as animal feeds in which the presence of tannins reduces digestibility (Crépon et al., 2010). Low tannin faba beans have pure white flowers, and pale seed coats. The expansion of flower colours into both the human consumption market and ornamental market would increase tannin levels. Flower classification is not only based on colour, but also on pattern. In faba bean, the wing spot has varying degrees of colour intensity, from entirely absent to filling the entire wing tissue of the flower. The standard can also be solid colour, or have variation in patterns. There are many known colours of faba bean including: white, brown, pink/brown, red, yellow spot, diffused yellow and pink/yellow (Cabrera, 1988), although the inheritance of some of these is unknown. The objective of this experiment was to (i) confirm the genetic control of the red flower phenotype and (ii) confirm the genetic control of the yellow wing spot phenotype.

Hypothesis i: Red flower colour is a double recessive phenotype, controlled by two genes.
Hypothesis ii: Yellow wing spot is controlled by a single recessive gene.

4.3 Material and Methods

For information about the initial crossing block and population development, see Chapter 3.0

4.3.1 F1 Growth & Testcrossing

The seeds harvested from plants of the selected crosses were harvested and planted on June 24, 2017 (for F1 seed development, refer to section 3.2). These F1 seeds were scarified and inoculated before being planted in 2 and 3 gallon pots in a phytotron chamber at the University of Saskatchewan under the same conditions as previously described (Section 3.1 and 3.2.1). Each pot was labelled with its respective female and male parents.

4.3.2 Phytotron F2 Plant Conditions & Flower Phenotyping

A phytotron chamber in the College of Agriculture and Bioresources at the University of Saskatchewan was used to grow 800 F2 plants that segregated for flower colour. An additional 20 parental lines were prepared for test crosses by seeding them into 10-cm pots using soil that was inoculated with *Rhizobium leguminosarum* bv. *viciae*. Plants were watered as needed and fertilized with 15-30-15 (N-P-K) fertilizer weekly after the first 4 weeks. The phytotron chamber provided 18 h daylength at 21 °C during the day and 18 °C during the night. The plants were seeded on December 8th, 2017 and harvested April 10th, 2018. The flower tissue colour was scored for each plant by recording the standard colour, wing colour and the presence or absence of a wing spot.

4.3.3 Field F2, F2:3 and Flower Phenotype Scoring

A field plot design was developed to grow the remaining F2 seeds, test cross seeds, and the harvested F2:3 seeds. A total of 9000 seeds were organized into microplots and seeded May 16, 2018. There were 3 rows per microplot, and sown at 14 seeds/row, 7.5 cm seed spacing, and 5 cm depth with plot separation of 121 cm. The microplots were divided into groups based on pedigree. The F2 populations that had Disco/2 as a parent were covered with a small bee-proof polyhouse to preserve the genetic integrity of the low vicine-convicine (LVC) trait by reducing the risks of outcrossing. The F2:3 seeds were grouped by pedigree and then sown in single plant derived rows. The testcross seed was grouped by cross and seeded in rows. The flower colour was scored for each plant by recording the standard colour, wing colour and presence of a wing spot. The F2s and

test crosses were scored individually, and the F2:3 generation was scored by row, indicating if the plants were segregating for flower colour or not. The flower colour of heterozygous F2:3 rows was recorded in relative proportions. Marrowfat peas were seeded between the different populations to separate them within the field design (F2, F2:3 and test crosses).

4.3.4 Statistical Analysis

A Pearson chi-square test of phenotypic distribution was performed for each F2 population to determine inheritance of the red flower and yellow wing spot. Additional Pearson chi-square tests were performed to determine inheritance of the other flower colour phenotypes observed. Phenotypes were categorized based on standard colour, solid or spotted wing pattern, and overall flower colour. Each population was analyzed for single, and double gene models for standard colour, wing colour and wing pattern. The segregation ratio was based on the number of independently assorting genes (Figure 4.1). One population was evaluated as a trihybrid.

Table 4.1: Expected Mendelian F2 segregation ratios for monohybrid, dihybrid and trihybrid gene assortment

Number of independent genes	Expected segregation ratio
1	3:1
2	1:3:3:9
3	27:9:9:9:3:3:3:1

(Mendel, 1865)

4.4 Analysis and Results

Before analysis, data were partitioned based on floral organ. To test the hypotheses of qualitative Mendelian genetics, a Pearson chi-square goodness-of-fit analysis was done. A Pearson chi-square goodness-of-fit test determines the probability that the observed data is occurring due to chance. For simplicity, this test will be denoted a “chi-square” test for the rest of the document. Excel version 16.23 © Microsoft, 2019.

$$X^2 = \sum \frac{(o-e)^2}{e}$$

(Bowley, 2008)

o= observed (4.1)

e= expected

X^2= chi-square value

The standard cut-off for significance with chi-square analysis is p value >0.05. If the deviation between observed and expected results in a p value less than 0.05 the probability that the observed deviation is caused by chance is only 5%, therefore there must be another factor causing the deviation.

To test Hypothesis i, multiple populations were developed, all of which segregated for red flower colour. In Table 4.2, data for 12 segregating F2 populations are reported. Due to the large yield variation among the initial parent crosses, population sizes have wide variation. Of the 12 populations, 9 fit the 15:1 segregation ratio indicating that the red flower phenotype is controlled by two recessive genes. There are also 3 populations that have deviated from the 15:1 hypothesis, indicating that these populations were influenced by other factors (genetic or otherwise). Test crosses were performed by crossing the F1s back onto the red flowered parent (P47-1), to further test the double recessive gene hypothesis. Although the backcross populations were small datasets they all had p-values>0.05 (Table 4.2). Therefore, Hypothesis i, that red flower is a double recessive phenotype, was accepted.

Table 4.2: Chi-square results of phenotypic segregation ratios for red flower in faba bean for 12 F2 (15:1) and 4 backcross populations (3:1) with 1 degree of freedom. Note: P47-1 is the red flowered parent and all other parents are non-red

Flower Tissue Phenotype					
Cross	Non-red	Red	Expected Ratio	X^2	P
F2	**F2 plants**	**F2 plants**			
Aurora x P47-1	109	7	15:1	0.769	0.381*
Gelber x P47-1	60	1	15:1	2.213	0.137*
P47-1 x Gelber	310	8	15:1	7.568	0.006
CDC Snowdrop x P47-1	89	7	15:1	0.178	0.673*
P47-1 x CDC Snowdrop	187	10	15:1	0.463	0.496*
P47-1 x Disco/2	136	0	15:1	9.067	0.003
P47-1 x Aurora	76	14	15:1	13.300	0.000
IG 114476 x P47-1	289	22	15:1	0.360	0.55*
Rinrei x P47-1	38	3	15:1	0.080	0.778*
P47-1 x Rinrei	143	5	15:1	2.080	0.149*
P47-1 x NV153	376	23	15:1	0.161	0.689*
Disco/2 x P47-1	87	2	15:1	2.434	0.119*
Backcross	**F1 plants**	**F1 plants**			
(P47-1 x Aurora) x P47-1	2	2	3:1	1.333	0.248*
(Rinrei x P47-1) x P47-1	4	2	3:1	0.222	0.637*
(P47-1 x Rinrei) x P47-1	7	0	3:1	2.333	0.127*
(P47-1 x NV153) x P47-1	3	1	3:1	0.000	1*

*=p-value >0.05, null hypothesis is accepted (observed ratio did not significantly vary from expected)

In a second experiment, two distinct wing spot colour phenotypes were observed: brown spot and yellow spot. The absence of wing spot was also observed and classified as a white. In Table 4.3 the cross between brown spot and yellow spot fit a 3:1 ($p>0.05$), and therefore Hypothesis ii was accepted.

The two white wing phenotypes used to determine the genetic behaviour of the yellow wing spot gene were CDC Snowdrop and Disco/2. Both have white standards, white wings and are known to be controlled by *zt1* and *zt2*, respectively, which are independent recessive genes. Although these are two independent genes they express the same phenotype and interact the same with the single recessive gene for yellow wing spot. In Table 4.3, two populations yielded significant p-values ($p>0.05$) for 9:3:4 recessive epistasis. When both white and yellow genes are present as homozygous recessive the solid white wing is expressed. Therefore white is epistatic to yellow spot. Backcross populations also confirmed a 1:1 ratio for populations with both zt1 and zt2.

Table 4.3: Chi-square results of three segregation ratios: single recessive gene (3:1) for brown spot: yellow spot; recessive epistasis (9:3:4) for brown spot: yellow spot: white and backcrosses (1:1). These phenotypes describe the coloration of the wing petals of the flowers of the F2 faba bean populations.

Flower Tissue Phenotype							
Cross	Brown Spot	Yellow Spot	White	Expected Ratio	DF	X^2	P
F2	**F2 plants**	**F2 plants**	**F2 plants**				
Aurora x Gelber	61	19	n.a	3:1	1	0.067	0.796*
Disco/2 x Gelber	75	21	32	9:3:4	2	0.500	0.779*
CDC Snowdrop x Gelber	128	41	49	9:3:4	2	0.791	0.673*
Backcross							
(Aurora x Gelber) x Gelber	5	2	n.a	1:1	1	1.286	0.257*
(CDC Snowdrop x Gelber) x Gelber	4	3	n.a	1:1	1	0.143	0.705*
(CDC Snowdrop x Gelber) x CDC Snowdrop	4	n.a	5	1:1	1	0.111	0.739*

*= p-value >0.05, null hypothesis is accepted (observed ratio did not significantly vary from expected)
Note: Parent flower colours are: Aurora=Brown Spot, Gelber =Yellow Spot, CDC Snowdrop=White, and Disco/2=White
n.a= data not available; DF= Degree of freedom

The term ‘solid’ is used to describe a phenotype where the wing petal is totally pigmented or has no pigment, whereas ‘spot’ refers to the phenotypes with an observable wing spot (Figure 4.1). Chi-square analysis was used to determine the genetic control of the solid wing phenotype (Table 4.4). All 10 populations fit the segregation ratio of 3:1 for solid wing: spotted wing. This confirms that the solid wing phenotype is controlled by a single recessive gene. This gene controls the spot pattern on the wing petal, rather than petal colour like the other genes discussed previously.

Table 4.4: Chi-square results of a single recessive gene (3:1) with 1 degree of freedom for spotted: solid wing petals. Phenotypes describe the colour pattern on faba bean wing flower tissue from multiple F2 populations.

	Flower Tissue Phenotype				
Cross	Spotted Wing	Solid Wing	Expected Ratio	X^2	P
	F2 plants	**F2 plants**			
Disco/2 x Gelber	96	32	3:1	0	1*
CDC Snowdrop x Gelber	169	48	3:1	0.96	0.327*
P47-1 x Aurora	66	24	3:1	0.133	0.715*
Aurora x P47-1	93	27	3:1	0.4	0.527*
P47-1 x Gelber	244	64	3:1	2.926	0.087*
Gelber x P47-1	47	14	3:1	0.137	0.712*
IG 114476 x P47-1	241	69	3:1	1.243	0.265*
P47-1 x Rinrei	118	33	3:1	0.797	0.372*
Rinrei x P47-1	36	5	3:1	3.585	0.058*
P47-1 x NV153	304	93	3:1	0.525	0.469*

*= p-value >0.05, null hypothesis accepted (observed ratio did not significantly vary from expected)
Note: Spotted Wing= Gelber, Aurora, IG114476, Rinrei, NV153; Solid Wing= P47-1, CDC Snowdrop, Disco/2

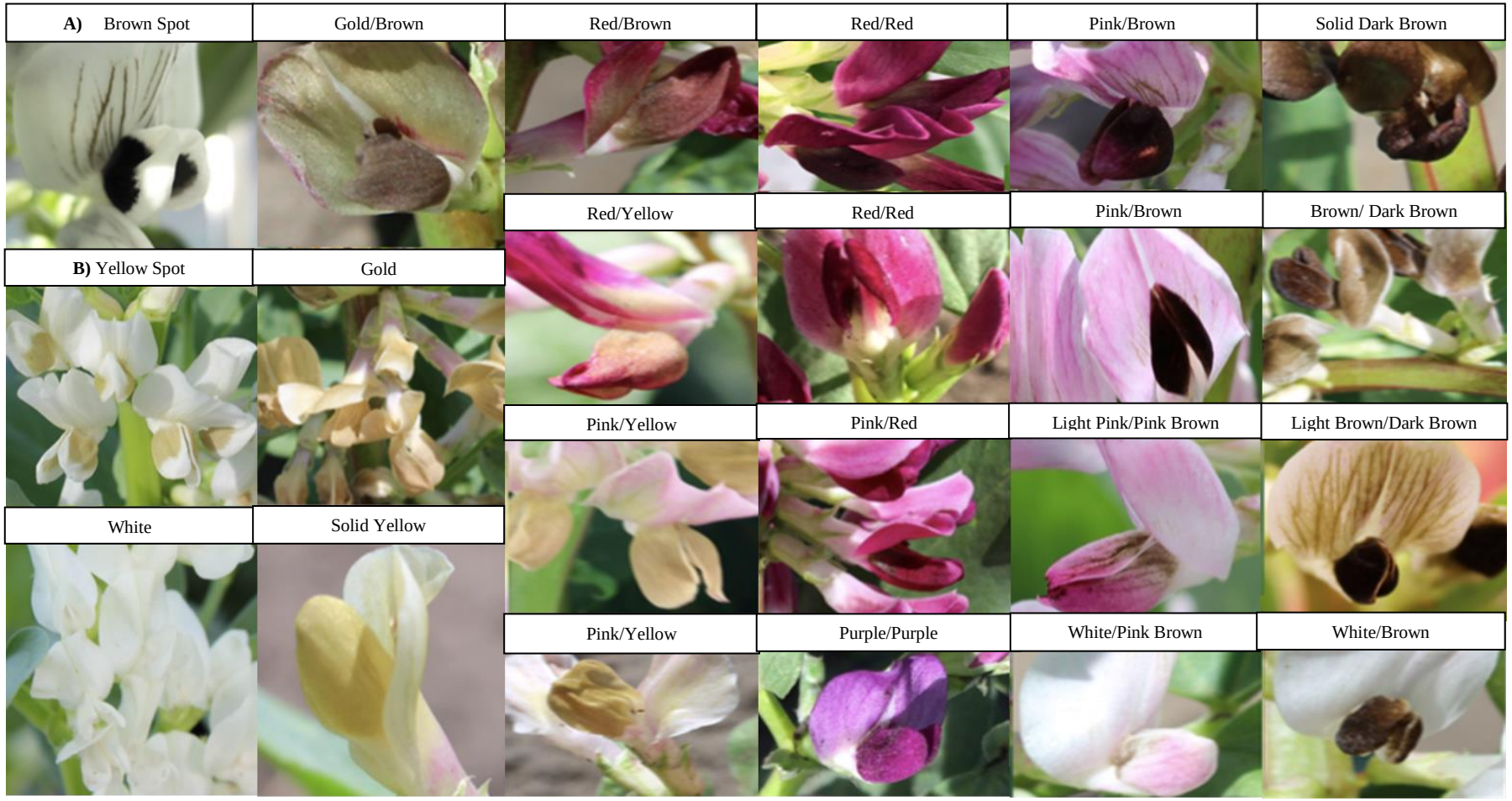

Figure 4.1: The full colour diversity observed among all F2 and F2:3 populations in these experiments. A & B are 'spot' wing phenotypes, whereas the rest are 'solid' wing phenotypes. Author's phenotypic nomenclature included.

4.5 Discussion

Throughout this study, multiple crosses and F2 populations were derived using contrasting parental lines. The population sizes were structured around Muller's formula (Table 3.3) with a 99% probability level, and the hypothesized gene models (Table 4.1). The comparisons between populations was possible due to the high percent of homozygosity in the parent lines (Table 3.2), which was derived from a comparison of polymorphic to monomorphic SNPs between all parent genotypes and P47-1.

4.5.1 Mendelian Segregation

Neither of the two flower colour hypotheses tested in this study were rejected. However, flower colour in *Vicia faba* is far more complicated than the two hypotheses originally postulated. The genetics of red flower colour had limited published work on inheritance. A publicly available red flowered faba bean called "Crimson" or "1778" was the initial source of the red flower used to develop P47-1, which was used in this study.

The yellow spot flower was a spontaneous mutation discovered by Rowlands in 1964. The F2 population segregating for the wild type: yellow spot phenotype fit a 3:1 ratio, and the backcross population fit a 1:1. This is consistent with the findings of Cabrera in 1988; however, the relationship between yellow wing spot and white wing had never been previously examined or reported. The yellow spot is controlled by a single recessive gene (Table 4.3). There are epistatic relationships with both *zt1* and *zt2* (Table 4.3). The yellow pigment appears to be a mutation of the brown pigment. My untested hypothesis for this observation is that the solid yellow flower is a brown flower with the recessive yellow spot gene. This hypothesis arose from a cross between a solid yellow flower with a solid red flower. Rather than the F1 plant having the wild type phenotype of white flowers with a brown spot (wild type), they were solid brown. This is consistent with the genotypes proposed in Tables 4.5 and 4.6.

Table 4.5: Genotypes proposed for P47-1 and Gelber along with the segregating trihybrid F2 plants

Proposed Trihybrid Genotypes		
Cross	Genotype	Phenotype
P47-1	*rrrrYY*	Red
Gelber	*RRRRyy*	Yellow Spot
P47-1 x Gelber	*R-R-Y-*	Brown Spot
P47-1 x Gelber	*rrR-Y-*	Solid Brown
P47-1 x Gelber	*R-rrY-*	Pink/Brown
P47-1 x Gelber	*rrR-yy*	Solid Yellow
P47-1 x Gelber	*R-rryy*	Pink/Yellow
P47-1 x Gelber	*rrrryy*	Red/Yellow

Table 4.6: Predicted test cross results for unique phenotypes and expected epistasis

Test Cross		
Cross Genotype	Cross Phenotype	F1 Genotype
rrrrYY x *rrRRyy*	Red x Solid Yellow	*rrRrYy*
rrrrYY x *Rrryy*	Red x Pink/Yellow	*RrrrYy:rrrrYy*
rrrrYY x *rrrryy*	Red x Red/Yellow	*rrrrYy*
rrrrZt2Zt2 x *rrRrzt2zt2*	Red x White	*rrRrZt2zt2*

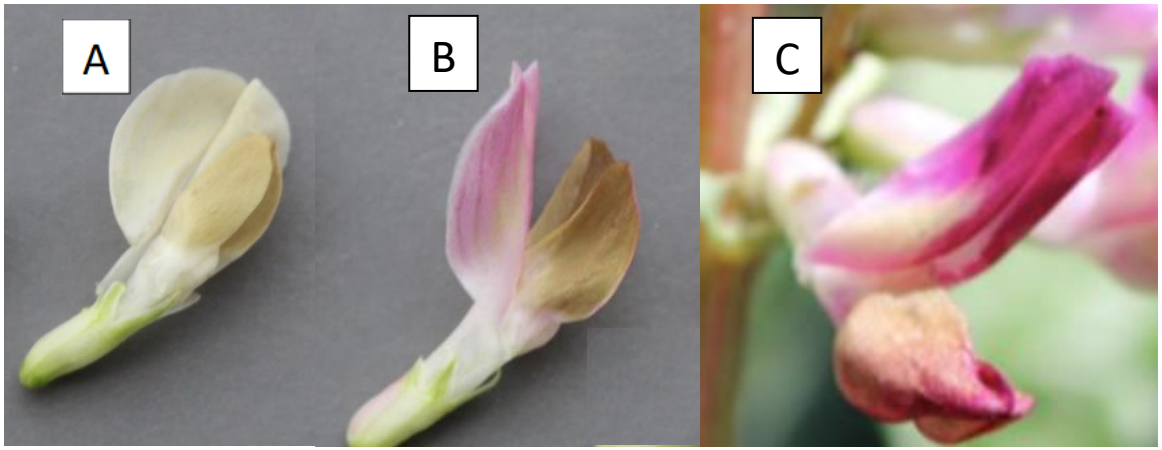

Figure 4.2: The rare phenotypes observed from the trihybrid cross of P47-1 x Gelber and the reciprocal. This is the first report of flower colour C. For the purposes of this thesis the phenotypes are titled: solid yellow=A, pink/yellow=B and red/yellow=C

4.5.2 Variation within phenotypic class

In addition to the relevant results of testing Hypothesis i and ii, there are more factors outside the scope of this research that influence the expression of colour in faba bean flowers. Faba bean flowers have colour variations within the same colour classes, and varying colour patterns. The standardized colour of the parent source of red flower in this study, according to the horticultural colour chart (Wilson, 1940), is actually Rhodamine purple 29/1. Very few of the red flowers in the F2 and F2:3 populations fit this same colour standard. As shown in Figure 4.1, there are gradients of colours within the same colour classes, with clear variation in intensity and hue. Within the red phenotype, for example, the pigmentation appears to changes hues from burgundy to purple. Some of these variations are undoubtedly genetic; however, others are possibly environmentally induced. Although the inheritance of the red flower fit the double recessive gene model, the red flowers of F2 plants were not identical in colour. Anthocyanins are a class of polyphenols responsible for purple pigmentation and are commonly upregulated as a result of stress (Oh et al., 2014). Therefore, some of the colour variation within this experiment could have been due to the high temperatures, high light intensity or even insect pressure under field conditions. The red flowered F2s within the same population also had variation in pigment expression; therefore, the red flower colour is influenced by more than just the presence or absence of the double recessive red alleles. The red flower phenotype derived from crosses with IG114476 and Rinrei is a cooler tone and appears purple; conversely, red flower types derived from an Aurora background have warmer red tones.

During this study the data collected were partitioned based on petal type, colour and pattern. From these data a 3:1 ratio for solid:spotted wing pattern was tested. Table 4.4 confirms that solid pattern is controlled by a single recessive gene. This result is also consistent with Cabrrera in 1988. The solid pattern was observed in the same colour diversity range that the standard petal exhibits; however, only two colours (brown and yellow) of the wing spot were observed. The last observable variation for flower colour was the petal pattern. Some of the phenotypes had distinct stripes (striae) running up the standard petal. However, since no data were collected on this specific expression, no tests for inheritance were performed.

4.5.3 Experimental Complications

Some of these populations had further complications due to trihybrid segregation, epistatic

relationships, and incomplete penetrance. The identification of the trihybrid nature among F2 progeny of the cross P47-1 x Gelber (and reciprocal) was determined by the higher than expected number of phenotypic classes than expected for a dihybrid. This study was designed to investigate a hypothesized monohybrid or dihybrid segregation ratio. As a result, the population sizes were not large enough to accurately test trihybrid segregation ratios of 27:9:9:9:3:3:3:1. Although Gelber x P47-1 fit the 15:1 gene model, it is not reliable due to the small population size and the trihybrid nature of the cross. P47-1x Gelber had an adequate population size but did not fit the 15:1 ratio due to phenotyping errors as a result of the large number of phenotypic classes. P47-1 x Gelber (red x yellow spot) has all the same phenotypes found in dihybrid crosses with P47-1, however it also has another set of phenotypes where the brown pigmentation was substituted for yellow. Test crosses were done to determine the genotypes of the unique phenotypes in this population (Table 4.6). A cross between a solid yellow flower phenotype (Figure 4.2A) and a red flower phenotype resulted in an F1 plant with dark brown flowers. Another cross between a pink/yellow flower (Figure 4.2B) and red flower resulted in a 1:1 ratio of red:pink/brown flowered F1's. These test crosses were consistent with the proposed genotypes in Table 4.5 and indicate the logical assumption that the phenotype expressing both yellow and red (Figure 4.2C), is the most recessive genotype. The red and yellow flower is a triple recessive, having both the double recessive red genes and single recessive yellow gene (*rrrryy*) for the trihybrid cross. The increased frequency of phenotypes and these test crosses strongly indicate a trihybrid. Therefore these populations are not reliable when evaluated as a dihybrid cross and should be re-evaluated. A trihybrid nature is also suspected for both CDC Snowdrop and Disco/2 (*zt1* and *zt2)* crossed with P47-1 since there are too many phenotypes expressed for a standard dihybrid cross (See Appendix Table A3). This explains why P47-1 x Disco/2 does not fit the 15:1 ratio. Although 8 easily distinguishable phenotypes were not observed (as in a trihybrid), there were many variations within each phenotypic class that could in fact be categorized as separate phenotypes. For example, in this study both light brown and dark brown were classified as one phenotype, when they could have been two independent phenotypes. In Table 4.3, *zt1* and *zt2* are independent from yellow spot, fitting a recessive epistasis model. This further suggests that *zt1* and *zt2* are independent from red, since they are also independent from yellow (Table 4.3).

Epistasis can cause one polyphenol to mask the expression of another, as was the case in a

Gerbera study in 1997 by Tyrach and Horn. Epistatic relationships affecting flower colour occur in many species, for example: safflower (Golkar et al., 2010), potato (Van Eck et al., 1993) and geraniums (Almouslem et al., 1991). According to an unpublished MSc. Thesis (Zanotto, 2018), faba bean is also known to have some epistatic relationships between the two white flower genes (*zt1* and *zt2*). This is consistent with the results in Table 4.3. Since epistatic relationships are known to occur in faba bean flowers it is also possible epistasis is influencing the full expression of phenotypic classes in the suspected trihybrid cross P47-1 x *zt1*/*zt2* (and reciprocal). A supplemental test cross was done after this experiment was concluded to determine if the trihybrid nature of P47-1 x Disco/2 was the sole reason this population did not fit the 15:1 ratio (Table 4.6). A white flowered F2:3 plant from P47-1 x Disco/2, was crossed with a red flower F6 and resulted in a pink and brown flowered F1 plant. This indicates that the genotype of the white flower phenotype in this cross had both a single recessive red allele and the recessive white (*zt2)*, but expressed a white flowered phenotype. This test cross indicated that the white flower is epistatic over at least one of the recessive red genes. This is a possible explanation to why no red flowers were observed in the F2 population of P47-1 x Disco/2 (*rrrr* x *zt2zt2*) in Table 4.2. Therefore deviation from the expected ratio is due to epistasis and the trihybrid nature of this cross.

The last population that did not fit the 15:1 double recessive gene model was P47-1 x Aurora. Although the reciprocal population (Aurora x P47-1) did fit the 15:1 ratio for non-red: red (Table 4.2), neither population had the expected number of phenotypic classes. The F2 population from Aurora x P47-1 fit a 12:3:1 ratio for dominant epistasis ($p=0.866$, $p>0.05$), however P47-1 x Aurora did not ($p=0.005$, $p>0.05$). The F2 from P47-1 x Aurora cross fit a recessive epistatic ratio of 9:3:4 ($p=0.077$, $p>0.05$) instead of dominant epistasis (See appendix A1). Another possible cause for the inconclusive result in the analyses that involve the parent line Aurora, is the heterozygosity rate (Table 3.2). If the parent line Aurora was heterozygous for genes that impact the expression of flower colour, unpredictable segregation would occur. The inconsistencies in these populations are inconclusive and need further research.

Another interesting pattern observed is the unique behavior between reciprocal crosses with P47-1, shown in Table 4.2. In crosses with Gelber, Disco/2 and Aurora as the female and P47-1 as the male the segregating F2 populations fit the 15:1 ratio for a double recessive gene model. In contrast, when P47-1 was the female for the same crosses, the F2 populations did not

fit the 15:1. Reciprocal crosses are the most direct way to test unequal contributions from maternal or paternal parents (Roach and Wulff, 1987). These populations are not simple dihybrids; therefore, they were not expected to fit a dihybrid ratio. However, it is still interesting that reciprocal crosses have distinct differences. This could indicate that maternal or paternal inheritance is affecting flower colour. Paternal inheritance is much less common than maternal. Maternal inheritance is not uncommon in plants and is known to be a factor across many traits in faba bean, such as plant height, seed weight, and seed yield/plant (Ghareeb and Fares, 2016). Maternal inheritance is often expressed in the F1, which was not the case in this study. To accurately determine the inequality in genetic contributions to these populations, additional research is required.

Many of the populations in this study were trihybrid or had epistatic relationships occurring. These unaccounted for genetic variables explain why some of the F2 populations did not fit the expected 15:1 segregation ratio for the double recessive gene model. The remaining populations, aside from the complications explained previously, had their own intricacies. The populations derived from P47-1 x NV153/Rinrei/IG 114476 were not trihybrids for flower colour nor did they have obvious epistasis, but they had their own deviations from expected segregation patterns. None of these populations fit a 9:3:3:1, for unknown reasons. All populations had an under expression of the intermediate phenotypes (brown and pink/brown). This could be a result of pleiotropy, which is known to occur for other traits in Rinrei (Fukuta et al., 2004). Although these populations fit the 15:1 ratio for a double recessive red genotype, the intermediate colours are more complex. None of the tested populations fit a 9:3:3:1 segregation - gene order or additivity could be potential explanations. Incomplete penetrance could also be influencing expression of flower colour causing the lower than expected intermediate colours. Further studies should be done to identify a marker with allelic discrimination to determine the genotype of the phenotypes segregating in these populations.

4.5.3 New Flower Colour Phenotype

Throughout the crosses, many colours and patterns were recorded (Figure 4.1). More rare phenotypes were discovered (Figure 4.2) by combining the yellow spot mutation with the double recessive red flower. Previous reports indicate the existence of the solid yellow flower, and the pink and yellow flower (Cabrera, 1988); however, there are no reports of the existence of the red

and yellow flower. This phenotype arose from a trihybrid cross between a red (P47-1) flowered plant and a yellow spot flowered plant (Gelber). The red and yellow flower occurs only in the complete homozygous recessive genotype from the trihybrid, which is expected at a frequency of 63:1 genotype in a trihybrid (Table 4.5).

4.6 Conclusions

Red flower and yellow wing spot are controlled by double recessive and single recessive genes, respectively. Although this explanation seems straight forward, faba bean flower colour is influenced by many other environmental and genetic. Faba bean flower colour varies in intensity, hue and pattern, which are influenced by both the environmental stresses causing anthocyanin upregulation, as well as genotype specific gene interaction with colour. Additionally, flower colour in many of the observed populations segregated for three genes, making them a trihybrid. Trihybrids are much more difficult to classify due to the large population size required to observe 8 potentially distinct phenotypic classes. Faba bean flower colour also has epistatic genetic relationships occurring between colours and within specific populations. This results in the masking of certain phenotypes, making phenotypic classification even more challenging. Although flower colour in faba bean has many complex characteristics, this study was not only able to determine the inheritance of two unique faba bean flower colours, but was also able to discover a new flower colour phenotype. The red and yellow phenotype has never before been reported, likely due to its highly homozygous recessive state.

CHAPTER 5. GENETICS OF PLANT ARCHITECTURE

5.1 Abstract

Plant architectural traits strongly influence plant success and influence which markets are most suitable. Faba bean in North America is primarily a minor grain legume, but in many areas of the world it is consumed as a fresh vegetable. A better understanding of plant architecture, including height and branching, could improve varieties for both current markets and potentially new ones. Two sources of dwarfism, NV153 and Rinrei, were used in this study. Segregating F2 populations were tested for 3:1 single recessive gene inheritance using chi-square tests. Both dwarfing genes fit 3:1 recessive ratios based on visual assessment, and 15:1 for double recessive. These genes are independent and additive. However, the Rinrei-type dwarfs did not fit the 3:1 model based on stem measurements. Rinrei is therefore not a true dwarf, and the gene creating the dwarf appearance reduces the initial growth rate, but this corrects over time. Multiple F2 populations were also created to test a 3:1 single dominant gene hypothesis for the highly branched phenotype. These populations had a normal bell-shaped distribution on a histogram, with no discernable classes. Therefore, it was concluded that branching is quantitatively controlled by multiple genes.

5.2 Introduction:

Dwarf plants are commonly used in agricultural systems as a method of improving agronomic traits. In wheat, dwarfism was used to reduce lodging and improve yields during the green revolution (Hedden, 2003). Aside from large scale agriculture, dwarfism is also used in more intensive production systems, such as the ornamental market. Smaller plants are easier to grow, harvest and maintain; therefore, dwarf varieties of many horticultural plants exist, from fruit trees to landscaping grasses. Understanding the dwarfing genes for faba bean is a key component to facilitate the expansion of faba bean into the ornamental market. Along with height, branching is both an important agronomic trait and an ornamental characteristic. Branching in large scale agricultural systems improves competitiveness by shading out weeds between seed rows. This also improves light absorption by increasing the canopy leaf area. Similarly, many ornamental plants are sold as ground cover to reduce weed pressure in landscaping. Understanding these key architectural traits will help breeders to diversify the markets for faba bean, and to develop plants for the ornamental market. The objectives of these

investigations were to determine the inheritance of two separate sources of dwarf faba beans, and determine the inheritance of the highly branched phenotype.

Hypothesis iii: Dwarfism and semi-dwarfism (sourced from NV153 and Rinrei) in faba bean are controlled by two independent recessive genes that can be combined.
Hypothesis iv: The highly branched faba bean phenotype is controlled by a single dominant gene.

5.3 Materials and Methods

For initial materials and methods, see chapter 3.0.

5.3.1 Field Grown F2 Population

To accurately test height and branching, plants need equidistant spacing. To ensure natural light conditions and equal spacing requirements were met, the 7 F2 populations segregating for dwarfism and 5 F2 populations segregating for branching traits were seeded into 12 cm deep Rootrainer ™ (Gardener's Supply Company, Burlington USA) transplant containers on April 16th 2018. From May 9-16th, the 1800 transplants were hardened off outside during the day, and were covered with a tarp at night to avoid frost risk. Black plastic mulch was laid with a Plastic Mulch Layer Model 560 (Rain Flo Irrigation, East Earl USA). On May 16th 2018, the young plants were transplanted into black plastic mulch with 25 cm spacing between plants in the field with a Vegetable Transplanter Model 1400 (Rain Flo Irrigation, East Earl USA) on May 16th 2018 (Figure 5.1). The transplants were fertilized with 20-20-20 (N-P-K) and then watered for 15 h with an underlain drip tape to reduce transplant shock. In-season watering and fertilizer was applied biweekly as needed. The transplants grew for a total of 121 d before being pulled from the ground for measurement of plant morphological characteristics.

Figure 5.1: A subset of Rinrei x NV153 F2 plants 26 days after being transplanted into black mulch at the Crop Development Centre, Saskatoon SK.

5.3.2 Phytotron Grown F2 Population

An additional location of this F2 experiment was performed in the phytotron in the fall of 2018, at the University of Saskatchewan. The F2 seeds were planted in milk crates (12 “x 12” x 10.5”) lined with polypropylene grain bags filled with Sun-Gro potting mix No. 3. The plants were seeded at a density of 4 plants/container. Light and temperature conditions as well as insect control was the same as described in Chapter 3. These plants were seeded on October 5th 2018 and grew for 96 d before being pulled and measured.

5.3.3 Plant Measurement Methods

Four measurements were recorded for each plant in both the field and phytotron experiments: plant height, number of branches, number of nodes and internode length. All plants were pulled from the soil and laid on a flat surface before measuring for better visibility. Main stem height was recorded for each plant by measuring from base to apex. The number of branches was determined by counting all branches originating from the main stem - secondary

branching was excluded. The number of nodes were counted on the main stem, beginning from the basal node to the stem apex. The average internode length was then calculated by dividing the stem height by the number of nodes. Aside from physical measurements, visual measurements were also recorded to classify the dwarf types into the visually distinct categories classified on the basis of leaf colour, leaf shape, and canopy structure.

5.3.4 Statistical Analyses

Chi-square tests were performed to determine the inheritance of the dwarfing characteristics of multiple F2 populations. Parameters were set based on the measurements of the parental lines of the segregating populations (Appendix B), and the visible groupings on the histograms (Figure 5.2-5.7, RStudio version 1.1463). These categories were then used to partition data into the qualitative categories appropriate for testing single gene hypotheses. A chi-square analysis was then done to determine if the observed data fit the expected Mendelian ratio for each hypothesis. Chi-square analysis was not done on the branching data because no discrete categories were discernible. To better visualize the data, histograms were made for each measurement in each population. The parent measurements were labelled on each histogram for classification.

5.4 Results

5.4.1 Dwarfism F2 Populations

The data were collected and then analysed in comparison to the parent controls for each data set. Parameters were set based on the parental line measurement data and visual scoring. Since the presence or absence of dwarfism is visually apparent, data partitioning was not a challenge. Histograms were also used to set parameters when distinct bimodal distributions were available. The hypothesized dwarfism gene in Rinrei did not show a bimodal nature on any height/internode length histogram (Figure 5.5-5.7). Therefore, the pleiotropic nature of the Rinrei dwarfism gene was used alongside the parental line for classification. Reciprocal crosses were attempted but only were successful with Rinrei as the female, due to the inbreeding nature of NV153.

5.4.1.1 Rinrei x NV153

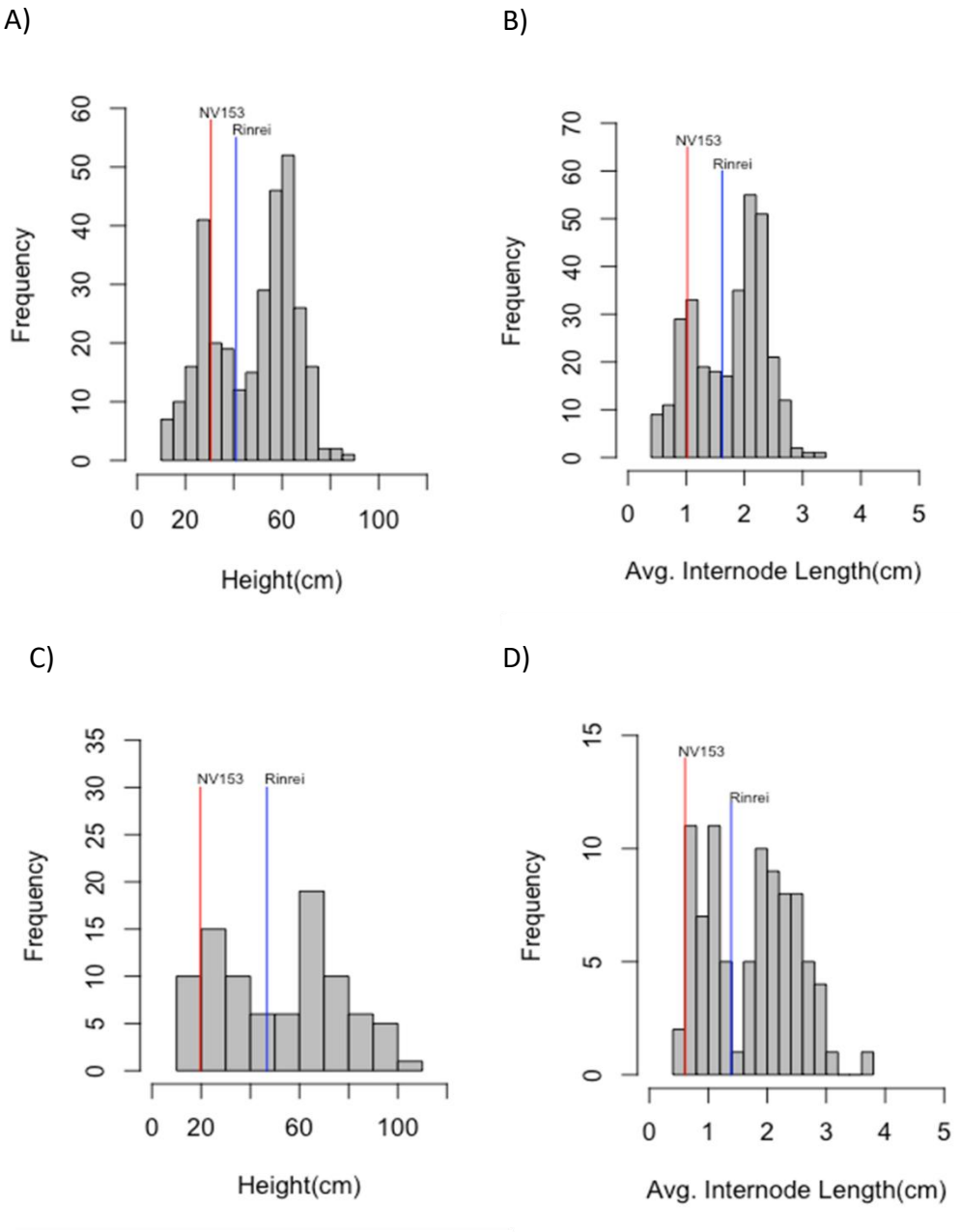

Figure 5.2: Height and average internode length distribution of the main stem for the F2 population of Rinrei x NV153 grown in field (A & B) and phytotron (C & D) conditions. Parent line measurements are in red and blue. N=314 Field and N=88 Phytotron plants

5.4.1.2 Aurora x NV153

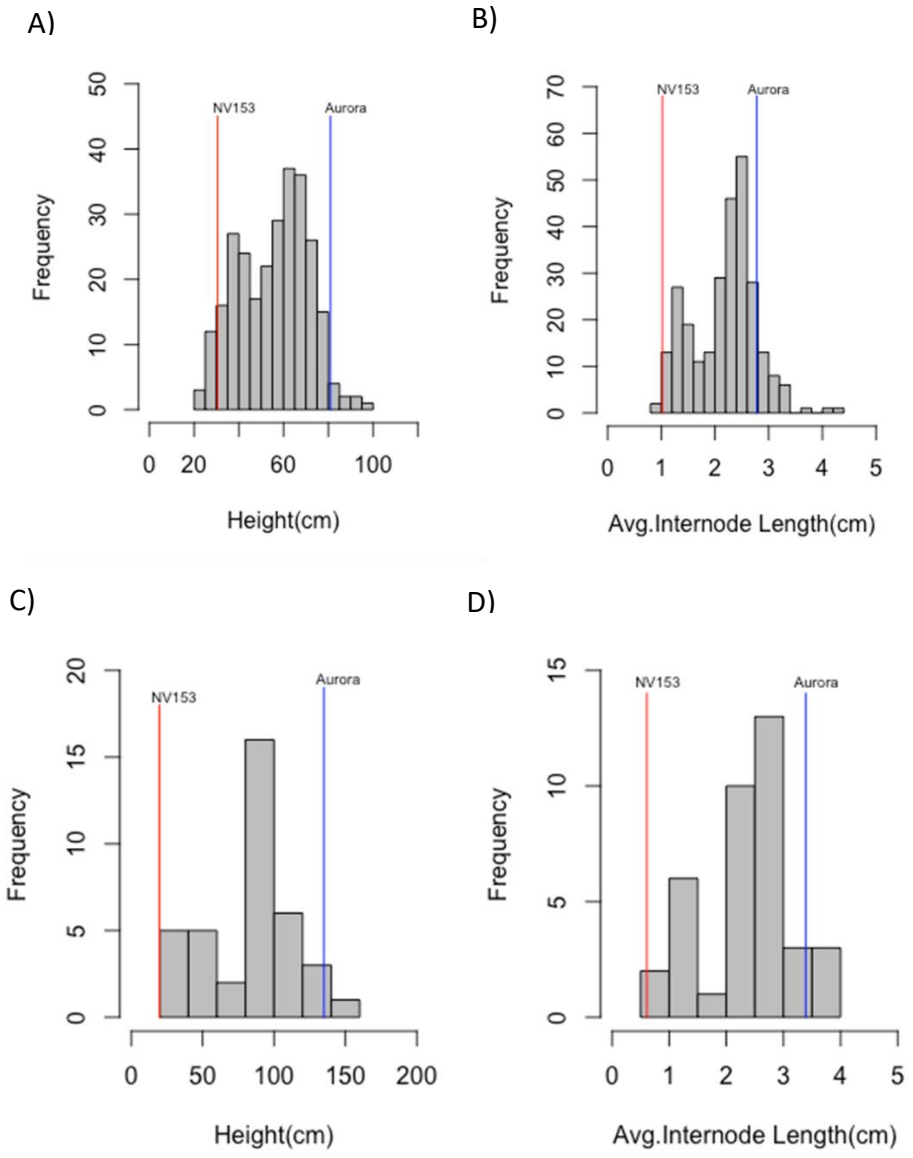

Figure 5.3: Height and average internode length distribution of the main stem for the F2 population of Aurora x NV153 grown in in field (A & B) and phytotron (C & D) conditions. Parent line measurements are in red and blue. N=273 Field and N=38 Phytotron plants

5.4.1.3 IG114476 x NV153

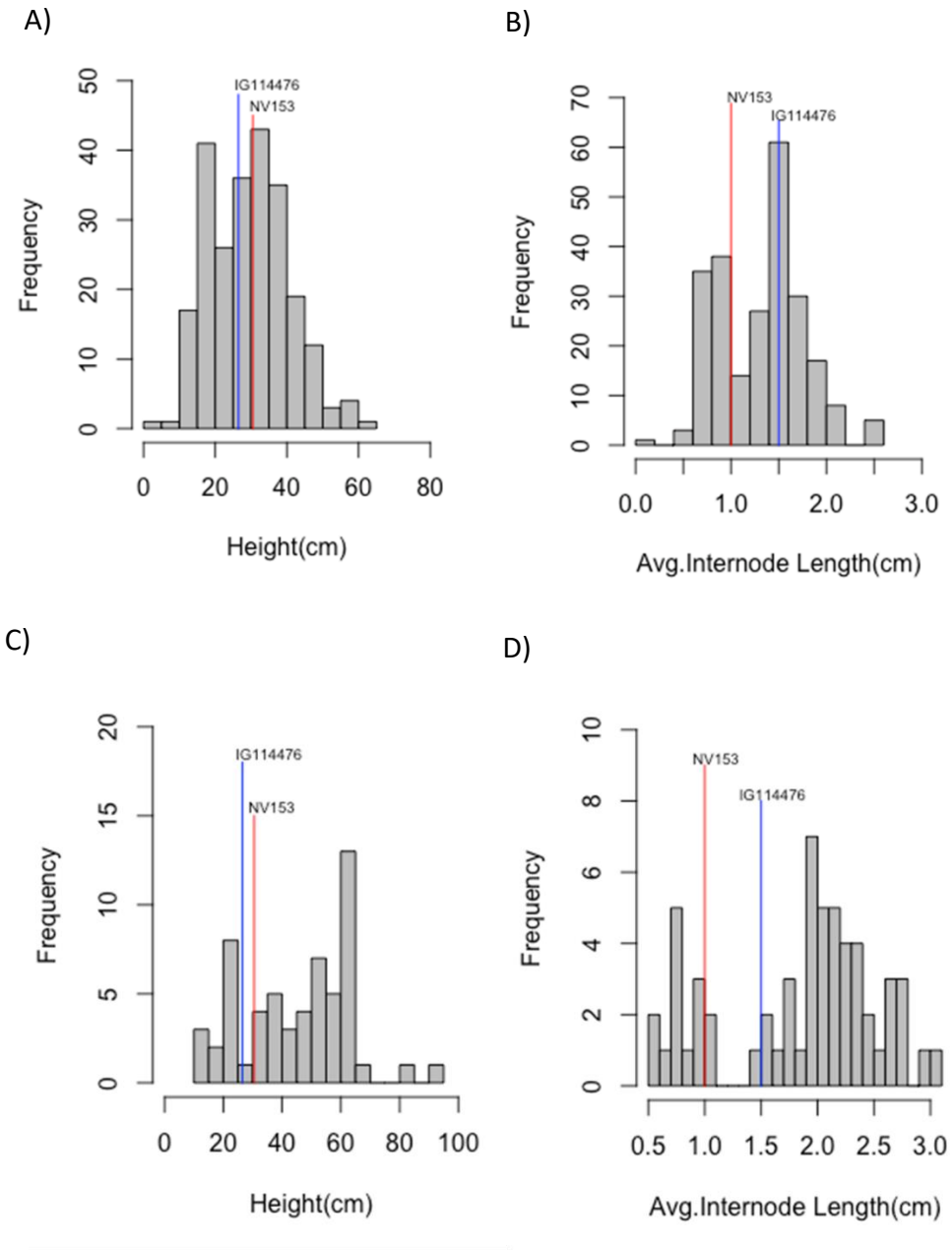

Figure 5.4: Height and average internode length distribution of the main stem for the F2 population of IG114476 x NV153 grown in field (A & B) and phytotron (C & D) conditions. Parent line measurements are in red and blue. N=239 Field and N=61 Phytotron plants

5.4.1.4 Rinrei x Aurora & P47-1 x Rinrei

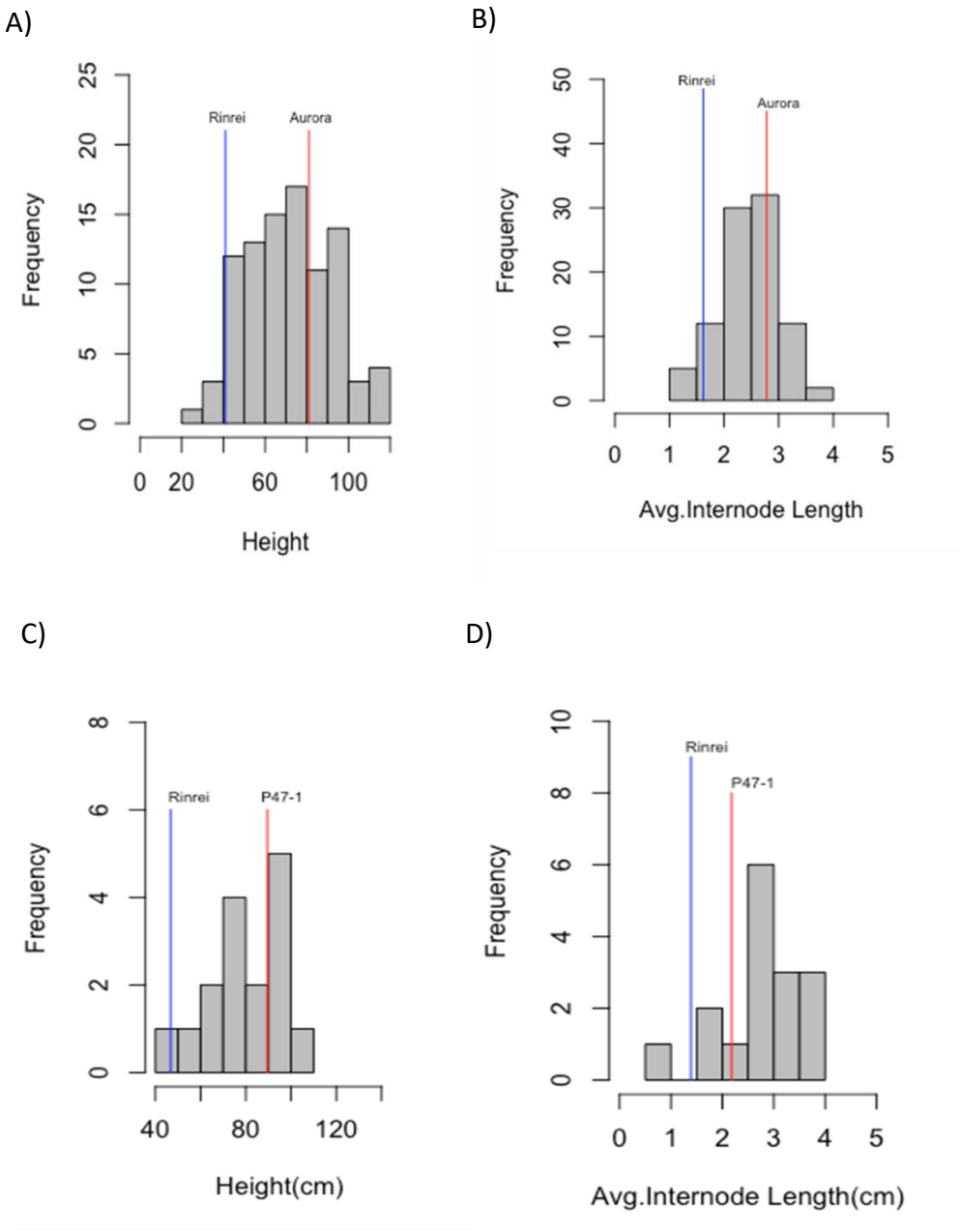

Figure 5.5: Height and average internode length distribution of the main stem for the F2 population of Rinrei x Aurora grown in field (A & B) and P47-1 x Rinrei in phytotron (C & D) conditions. Parent line measurements are in red and blue. N=93 Field and N=16 Phytotron plants

5.4.1.5 Rinrei x IG114476

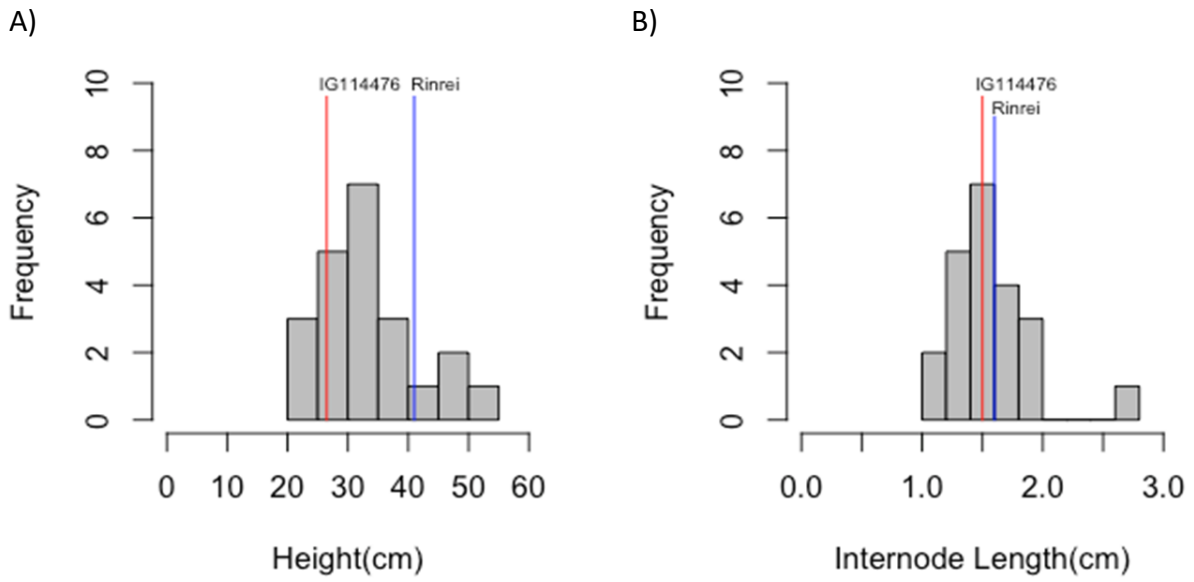

Figure 5.6: Height and average internode length distribution of the main stem for the F2 population of Rinrei x IG114476 grown in field conditions (A & B). Parent line measurements are in red and blue. N=44 Field plants

5.4.1.6 IG114476 x Rinrei

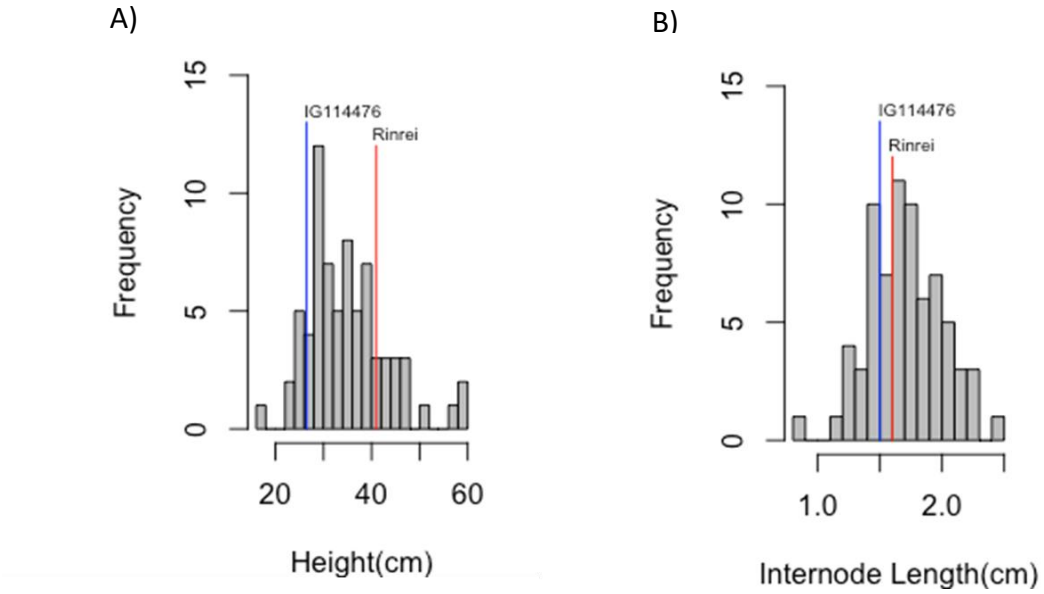

Figure 5.7: Height and average internode length distribution for the F2 population of IG114476 x Rinrei grown in field conditions (A & B). Parent line measurements are in red and blue. N=144 Field plants

Table 5.1: Chi-square results of 7 F2 populations segregating (3:1) for two independent recessive dwarfism genes in faba beans with 1 degree of freedom. Note: NV153 and Rinrei are the two dwarf parent lines. The Rinrei x NV153 population is tested twice, once for each hypothesized dwarfing gene.

Generation		Number of Plants Observed							
F2	Location	Normal	Rinrei Dwf	NV153 Dwf	Expected Ratio	X^2	P	Comb.X^2	Comb. P
Aurora x NV153	Field	194	n.a	79	3:1	2.258	0.133*	1.467	0.226*
	Phyto	30	n.a	8	3:1	0.316	0.574*		
IG114476 x NV153	Field	168	n.a	71	3:1	2.824	0.093*	3.004	0.083*
	Phyto	44	n.a	17	3:1	0.268	0.605*		
IG114476 x Rinrei	Field	94	50	n.a	3:1	7.259	0.007	5.274	0.022
	Phyto	12	2	n.a	3:1	0.857	0.355*		
Rinrei x NV153	Field	232	n.a	82	3:1	0.208	0.648*	0.030	0.863*
	Phtyo	68	n.a	20	3:1	2.97	0.085*		
Rinrei x NV153	Field	244	70	n.a	3:1	1.227	0.268*	0.030	0.863*
	Phtyo	59	29	n.a	3:1	2.97	0.085*		
Rinrei x Aurora	Field	75	18	n.a	3:1	1.581	0.209*	n.a	n.a
P47-1 x Rinrei	Phyto	14	2	n.a	3:1	1.333	0.248*	n.a	n.a
Rinrei x IG114476	Field	29	15	n.a	3:1	1.939	0.164*	n.a	n.a

Field= North Seed Farm at the Crop Development Centre, Saskatoon SK
Phyto= phytotron chamber at the University of Saskatchewan
Dwf= Dwarf ; n.a= data not available; Comb.X^2= Combined X^2; Comb.P= Combined P value
*= p-value >0.05, null hypothesis accepted (observed ratio did not significantly vary from expected)

Table 5.2: Chi-square results for a F2 population of faba bean testing the independence of the two recessive dwarfing genes (9:7), and the double dwarf hypothesis (9:3:3:1 & 15:1). Note: NV153 and Rinrei are both dwarf parent lines.

Generation		Number of Plants Observed									
F2	Location	Normal	Rinrei Dwf	NV153 Dwf	Double Dwf	Expected Ratio	DF	X^2	P	Comb.X^2	Comb. P
Rinrei x NV153	Field	182	132		n.a	9:7	1	0.374	0.541*	0.036	0.850*
	Phyto	46	42		n.a	9:7	1	0.566	0.452*		
	Field	182	50	62	20	9:3:3:1	3	1.674	0.643*	0.308	0.958*
	Phyto	46	22	13	7	9:3:3:1	3	3.232	0.357*		
	Field	294	n.a	n.a	20	15:1	1	0.008	0.930*	0.149	0.699*
	Phyto	81	n.a	n.a	7	15:1	1	0.436	0.509*		

Field= North Seed Farm at the Crop Development Centre, Saskatoon SK
Phyto= phytotron chamber at the University of Saskatchewan
Dwf= Dwarf ; n.a= data not available; DF= Degrees of freedom; Comb.X^2=Combined X^2; Comb.P= Combined P-value
*= p-value >0.05, null hypothesis accepted (observed ratio did not significantly vary from expected)

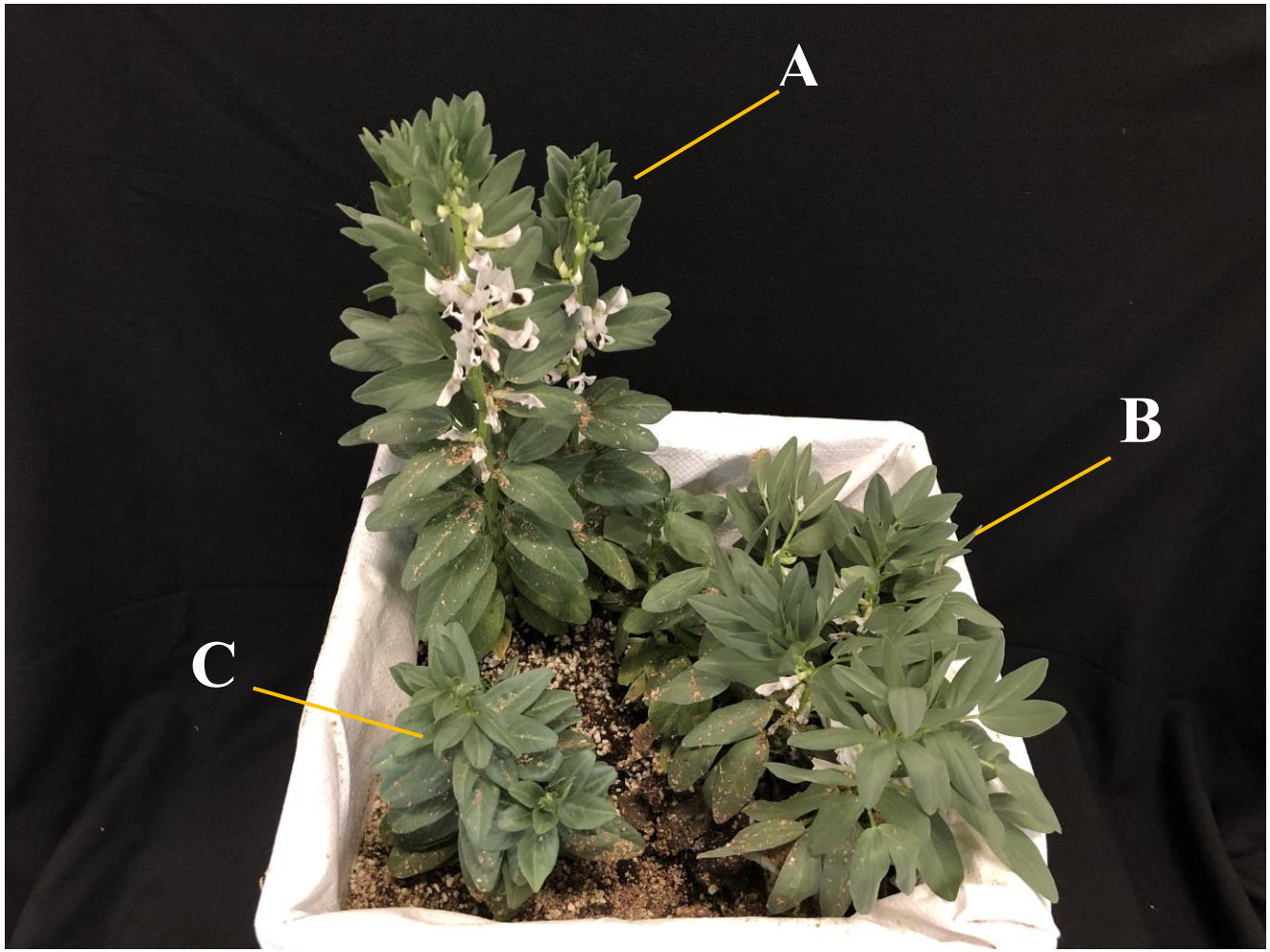

Figure 5.8: F2 population showing the 3 dwarf type plants phenotypes: Rinrei type (A), NV153 type (B) and Double Dwarf (C), grown in the phytotron at the University of Saskatchewan.

Multiple F2 populations were examined to determine the inheritance of the two dwarfing genes in this study. NV153 dwarf plants developed as expected for a single recessive gene, showing bimodal distribution on the histogram (Figure 5.2-5.4) with a significant p-value for a 3:1 chi-square analysis (Table 5). The Rinrei type dwarf measurements did not distribute as expected for a single recessive gene. Rinrei populations did not show a bimodal distribution on a histogram (Figure 5.5-5.7) for either height or internode length, but did fit a chi-square analysis based on the visual characterization of the Rinrei type F2 plants (Table 5.1 & 5.2). Additionally, the cross between Rinrei and NV153 resulted in a normal height F1 plant, indicating the two dwarfing genes in this study are independent genetically and physiologically. The F2 population from this cross did have a bimodal distribution; however, it was skewed to the right, indicating a higher than expected ratio of dwarf phenotypes than for a single dwarfing gene. Therefore, Hypothesis iii, the dwarfing genes are controlled by two independent recessive genes, was not rejected. The independent nature of these genes made it possible to create a genotype expressing both dwarfing genes (termed double dwarf for the purpose of this study) and a phenotype with extremely reduced height (Figure 5.8). The double dwarf phenotype segregated as a 15:1 ratio, fitting a double recessive model (Table 5.2).

5.4.2 Branching F2 Population

5.4.2.1 IG114476 x Aurora

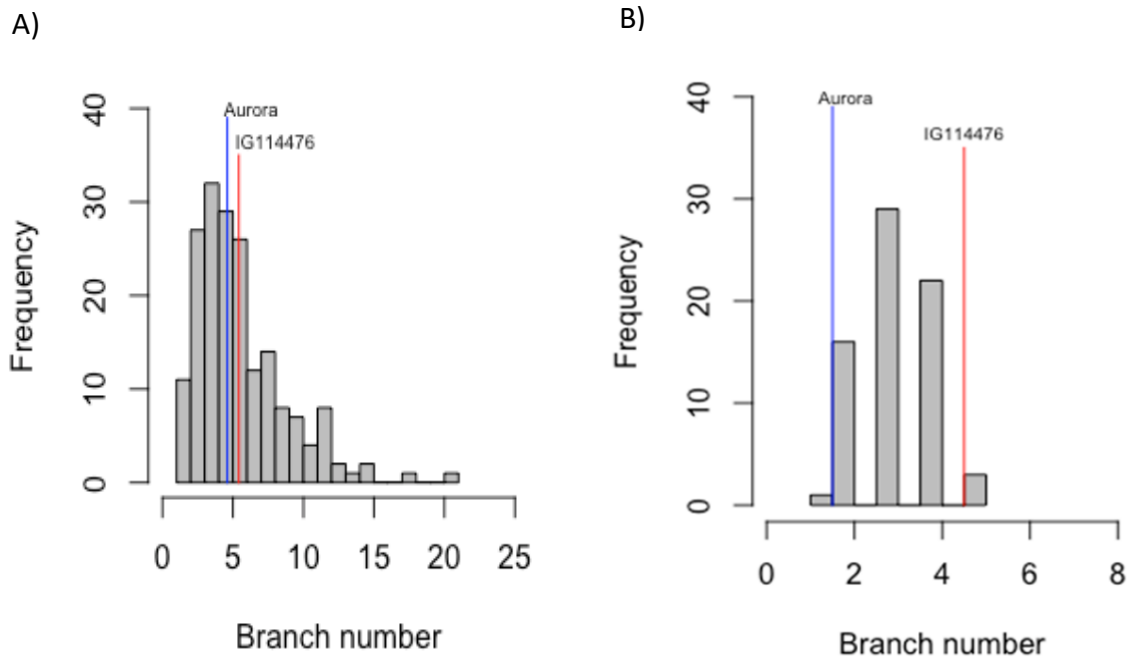

Figure 5.9: Branch number distribution for the F2 population IG114476 x Aurora grown in field (A) and phytotron (B) conditions. Parent line measurements are in red and blue. N=185 Field and N=71 Phytotron plants

5.4.2.2 Aurora x IG114476

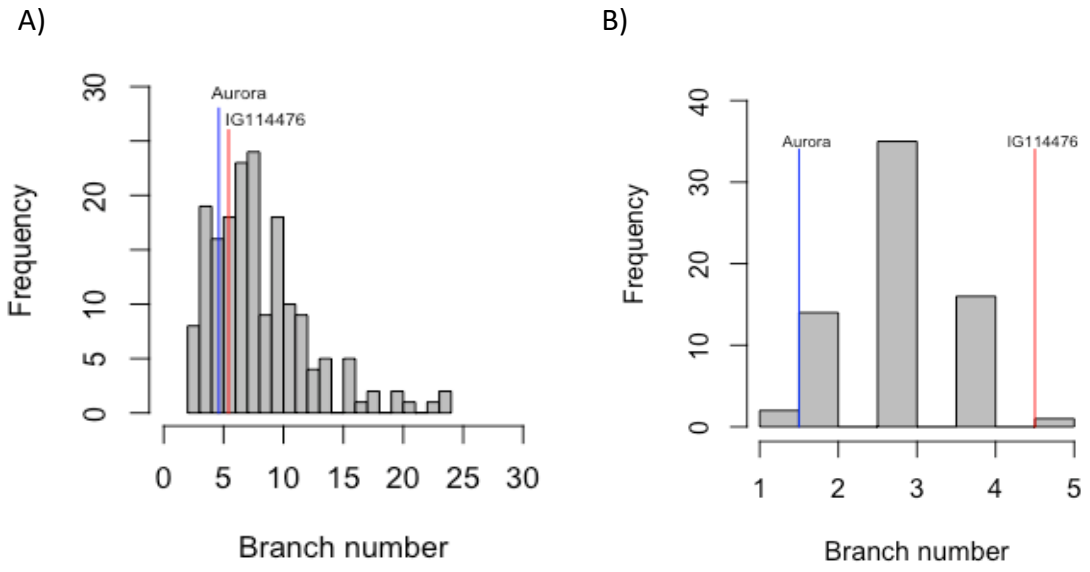

Figure 5.10: Branch number distribution for the F2 population Aurora x IG114476 grown in field (A) and phytotron (B) conditions. Parent line measurements are in red and blue. N=177 Field and N=68 Phytotron plants

5.4.2.3 IG114476 x NV153

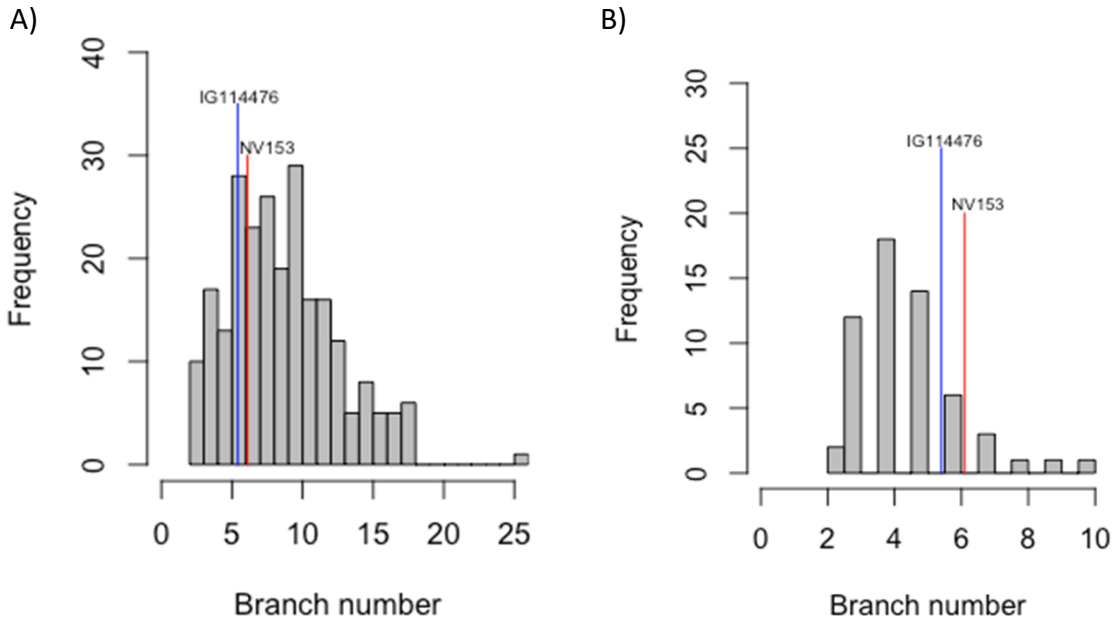

Figure 5.11: Branch number distribution for the F2 population IG114476 x NV153 grown in field (A) and phytotron (B) conditions. Parent line measurements are in red and blue. N=239 Field and N=61 Phytotron plants

5.4.2.4 IG114476 x Rinrei and Reciprocal

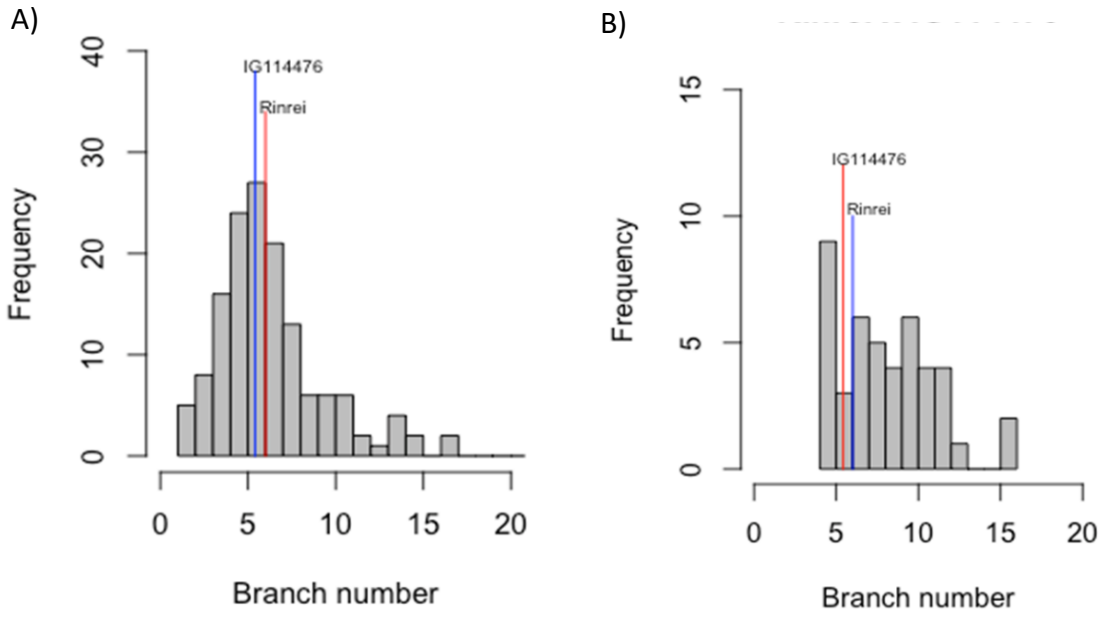

Figure 5.12: Branch distribution for the F2 populations IG114476 x Rinrei (A) and Rinrei x IG114476 (B) grown in field conditions. Parent line measurements are in red and blue. N=144(A) Field and N=44(B) Field plants

Multiple F2 populations were examined to determine genetic control of branching. These populations did not show discrete categories and had a normal distribution of branch number (Figure 5.9-5.12). Therefore Hypothesis iv was rejected, and branching was considered a quantitatively inherited trait. The inconsistencies for the graphs with non-normal distributions are the result of small population sizes.

5.4.3 Discussion

Dwarfism and branching are both architectural phenotypes that impact plant competitiveness and visual appearance. In faba bean, these two traits are inherited differently. Both dwarfing genes studied in this analysis fit a single recessive gene model and are therefore qualitative. In contrast, the segregation for branching was not discrete. Branching had a continuous segregation, which is indicative of quantitative inheritance and is therefore more difficult to manipulate from a breeding perspective.

Dwarfism is a well known and well used phenomenon in plant breeding. The ability to manipulate plant height can lead to increased yield, vigor and agronomic characteristics. It also can lead to the development of new products and markets, one of the intended aims of this body of research. In the analyzed populations the expression of dwarfism was consistent, regardless of the parent genotype crossed with the dwarf parental line. This indicates dwarfism is not influenced by either the physical, or the genetic environment, and consistently segregated 3:1.

Pleiotropy was first formally discovered in 1910 by Ludwig Plate, a German scientist. Pleiotropy is a natural occurrence where the presence of one locus affects the expression of two or more phenotypic traits (Stearns, 2010). Pleiotropy is common in both plants and animals. Pleiotropy has an impact on evolution (Albert et al., 2002), diseases (Pyeritz, 1989), syndromes and ultimately, death (Williams, 1957). Pleiotropy in this study was observed with the dwarfing gene from Rinrei. It conveys multiple phenotypic effects on the plant, including: dark green foliage, short stature, short petioles and thick stems. This is consistent with the conclusions of Fukuta et al. in 2004. The Rinrei expression is known to be caused by a brassinosteroid deficiency, which explains the reduction in cell expansion and elongation (Fukuta et al., 2004). However, in contrast to the results of Fukuta et al., this research identified the Rinrei dwarfing gene to be a slow growth gene, rather than a dwarf/semi-dwarf. This slow growth gene reduces the growth rate, causing the appearance of early season dwarfism, but the plants continue to grow and eventually no longer appear to be dwarfed. Figure 5.8 illustrates the unique growth habits of the different types of dwarfism in this study. NV153 has fine leaves and more of a bush shape architecture; in contrast Rinrei, has a very stiff stem and sharp vertical architecture. The dwarfism gene from NV153 has no obvious pleiotropic effects and segregates as expected for a 3:1. The combination of these two genotypes creates a very unique and interesting foliage and architecture (Figure 5.8 C).

The inheritance of the highly branched phenotype in this experiment was more difficult to characterize. Degree of branching is influenced by environment in faba bean (Abu-Amer et al., 2010). To account for that, the same populations were grown in two environments to best observe the highly branched trait. Branching was consistently higher when the plants were grown in field conditions in comparison to branching in phytotron conditions (Appendix B). This is also visible in Figures 5.9 and 5.10, where Aurora has a branch value of 1.5 in phytotron conditions, and 5 under field conditions. Interestingly, IG114476 has the most consistent branching regardless of the environment, indicating a strong genotype effect. An additional possibility that could be influencing these observations is the development of secondary branching. Secondary branching is characterized by production of new branches late in the growing season. Secondary branching is common in faba bean, and is more prominent in the winter sown types (Knott, 1990). Secondary branching can be identified based on the age of the branch. Rinrei is a winter sown type bean, and as shown in Figure 5.12, Rinrei appears to have more branches than IG114476. This result is likely due to the high degree of secondary branching in Rinrei. NV153 also appears to have more branches than IG114476 (Figure 5.11), indicating that this dwarf type should also be considered a highly branched type. Branching is an important trait for both agronomic and ornamental markets. Understanding the inheritance of branching aids in the development of more competitive varieties or more esthetic ornamentals.

5.4.3.1 Experimental Complications

A major challenge during this experiment was creating a consistent environment for all the plants within the same location. This was challenging in the phytotron because the F2 populations were segregating for dwarfism; however, at the time of sowing there was no way to predict which seeds would produce dwarf plants. This resulted in dwarf plants being shaded out by the normal height plants in the container growth system. Plant placement was optimized to reduce etiolation; however, some plants became etiolated. In contrast, the dwarf parent lines in the phytotron were seeded next to each other and therefore had much less competition for light. These variations caused characterization of the dwarfism parameters to be more challenging and left more room for potential error. In addition, the parent line checks for the outdoor experiment were planted at a different location, approximately 2 km away. This was a result of last minute changes in planting locations, causing a single experiment to be separated over two locations

without the addition of another set of parent line checks. The deviations between the parent lines and the F2 dwarf type plants were mediated by the visual characterization of the dwarf type plants, and an experiment replication in the phytotron at the University of Saskatchewan in fall 2018.

The black mulch that was used as ground cover and to make equidistant spacing (Figure 5.1) also created problems with heat control. Spring 2018 in Saskatchewan was very hot, and the black plastic mulch heated in the sun. In the days after the transplants were put in the field, the temperatures were between 25-30 °Cs. The plants with weaker stems that were laying on the black plastic mulch while recovering from transplant shock developed stem heat canker, which in turn cut off the xylem and phloem to the top of the shoots. This resulted in the loss of the main stem for many of the transplants. These plants had to be removed from the data set since the removal of the shoot apical meristem broke apical dominance and resulted in increased branching. This would have resulted in unreliable branching data, but also could have introduced a bias since the highly branched types were more likely to have contact with the black mulch and develop a heat canker. Black mulch should be replaced with a weed supressing white mulch in future experiments.

5.5 Conclusions

Dwarfism and branching are controlled qualitatively and quantitatively, respectively, in faba bean. Both sources of dwarfism in this experiment are controlled by independent single recessive genes, although the Rinrei type plants are not true dwarfs. These genes can be combined and cause an extreme height reduction. Branching is controlled by multiple genes and therefore could not be characterized within the scope of this experiment. In faba bean, dwarfism is not affected by environmental conditions, but branching is affected.

CHAPTER 6. LINKAGE ANALYSIS OF GENES FOR VICINE-CONVICINE AND HORTICULTURAL TRAITS

6.1 Abstract

Vicine and convicine (VC) are compounds found naturally in most genotypes of faba bean. They are potentially toxic to individuals with the glucose-6-phosphate dehydrogenase (G6PD) enzyme deficiency. It is estimated that 400 million people have this deficiency around the globe, making the reduction of VC crucial for the future of faba bean as a food source. To determine if the VC gene is linked to some of the economically important horticultural traits, F2 populations were developed and screened. Both the phenotype and corresponding VC genotype were determined and tested against a chi-square test ($p>0.05$). These tests confirmed there is no linkage between the VC marker and flower colour or dwarfism.

6.2 Introduction

There are many antinutritional compounds found in plant species, with varying effects on humans and animals. Vicine and convicine (VC) are antinutritional compounds found in faba bean. They are pyrimidine glycosides that hydrolyze into their algycone derivatives (divicine and isouramil, respectively) in the digestive tract (Jamalian and Ghorbani, 2005). These derivatives cause the hemolytic anemia known as favism in individuals with G6PD (glucose-6-phosphate dehydrogenase) enzyme deficiency (Cappellini and Fiorelli, 2008). Vicine and convicine exist in faba beans at varying concentrations dependent on genotype, and this diversity made breeding low VC (*vc*-) varieties possible, and eventually a molecular marker was developed for this trait. The low VC marker (Khazaei et al., 2017) facilitated the rapid screening of germplasm which improved breeding efforts for low VC varieties. The low VC type faba bean makes the expansion of faba bean into the human food market more feasible. This *vc*- genetic marker is well suited for KASP allele identification, which allows quick genetic screening of hundreds of samples quickly, and allows for bi-allelic scoring of SNPs in a single reaction. The reduction of VC in faba bean is crucial for expanding faba bean into plant protein and vegetable markets. Plant protein consumed as an alternative to animal protein is more sustainable and environmentally friendly (Sabaté and Soret, 2014). Since faba bean is such a high yielding, good quality protein source, it is a natural fit for the plant protein market. In addition to the protein market, low VC

vegetable type varieties would also facilitate expansion of production and consumption of the faba bean as a vegetable. The objective of this study was to determine if VC is genetically linked to any of the horticulturally important traits in this study.

Hypothesis v: There is no linkage between the low vicine-convicine marker and any of the qualitative genes that result in the desired horticultural phenotypes in this research.

6.3 Materials and Methods

For initial materials and methods, see chapter 3.0.

6.3.1 DNA Extraction and KASP Assay

Cotyledon tissue was extracted from the F2 seeds segregating for the low VC trait. Once the seed coat was chipped, ~5 µl of distilled water was dispensed onto the exposed cotyledon to soften the tissue. A wooden pick was used to scrape tissue from the cotyledon and place it into a PCR well plate along with 10 µl of water. Once the PCR well plate was full, 40 µl NaOH was added to each well. The plate was then sealed, mixed on a vortex and heated at 95°C for 2 min. Next, 60 µl of Tris-HCl (0.5M and pH of 8) was added, the plate was resealed, mixed and heated at 95°C for 2 min.

Once all the DNA for all the samples was extracted, the extractions were diluted 1:10 sample: water and then plated onto a 24 x 16 well KASP plate. Each well of the KASP plate contained 4 µl KASP master mix (FRET cassette (both VIC® and FAM™), taq polymerase enzyme and buffer solution), 2µl water, 2µl diluted DNA and 0.15µl allele specific primers for the KASP_*vcp2* marker (Khazaei et al., 2017). The plate was sealed using an adhesive sealer (BIO-RAD, USA) and run for 2 h 45 min through the PCR program for KASP assays developed by Robert Stonehouse at the University of Saskatchewan (Appendix C), as recommended by the KBiosciences in the KASP manual. This program includes a hot start to activate the taq polymerase and a 2-step touchdown in the first 10 cycles followed by 48 more cycles. An initial fluorescence image was taken before any PCR cycles. During the cycling, fluorescence images were taken at 5 time points. Once the KASP results were finished, they were standardized by eliminating the initial fluorescence values determined at the beginning of the KASP run.

6.3.2 Field Set-up and Phenotyping

The genotyped F2 seeds of crosses with Disco/2 as a female or male parent were grouped based on pedigree and allelic state for the LVC gene. The seeding specifications and plot parameters were the same as section 4.3.3. To reduce outcrossing and contamination of high vicine-convicine (HVC) with low vicine-convicine (LVC), two pollinator proof tents were constructed over the genotyped plants. One tent enclosed all the plants with the homozygous recessive genotype for LVC, while the other tent enclosed the heterozygous genotypes and homozygous dominant HVC genotypes.

6.3.3 KASP Data Setup

The KASP (Kompetitive Allele Specific PCR) output VIC® and FAM™ values for each sample of DNA. These values were standardized with the initial inflorescence image taken before any rounds of PCR. Fluorescence values were taken at 5 time points (P1, P2, P3, P4, P5), to obtain the most informative fluorescence value. Post 5 was primarily the time point used. Some of the fluorescence values were low after one run, and those plates were run again and time point 6 (P6) was used. A high VIC fluorescence value indicates the cassette binding to the *vc*- allele, and the sample has the LVC genotype. A sample with both VIC and FAM fluorescence values indicates that the cassettes for both alleles are present and therefore the sample is heterozygous for the VC gene. A sample with a high FAM value indicates the LVC gene is not present. The fluorescence value was calculated for each sample by subtracting the initial fluorescence from the fluorescence after each successive cycling step then divided by the initial fluorescence and multiplied by 100 to give the percent change in fluorescence. These values were then visualized on Cartesian plots where change in FAM is x-axis, and percent change in VIC is y-axis (Figure 6.1-6.3).

6.4 KASP Assay Results and Analysis

To analyze the data, Cartesian plots were developed based on the FAM and VIC values. The three groups were confirmed by the presence of LVC and HVC checks and controls. These groups formed visual clusters on the graph and are classified as: homozygous low, heterozygous and homozygous high for *vc*- (LVC, HET, HVC respectively).

6.4.1 Cartesian Plot Results

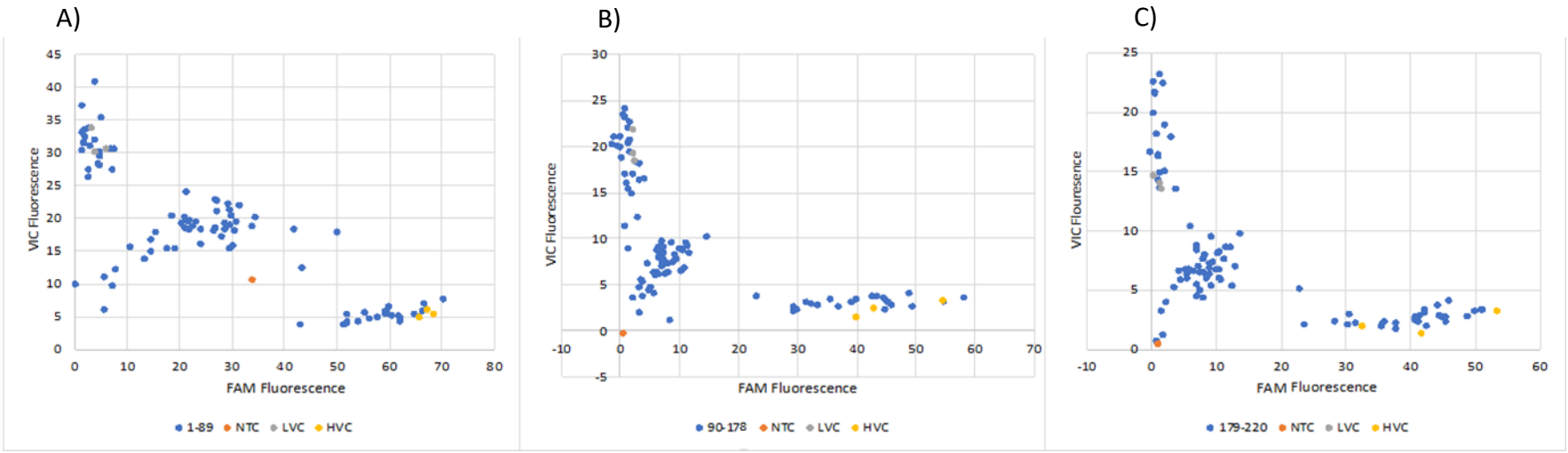

Figure 6.1: Cartesian plot for the allelic discrimination of *vc*- by KASP for the F2 population Rinrei x Disco/2. FAM fluorescence and VIC fluorescence are on the x and y axis respectively. This data was recorded at time point 6(A) and 5 (B & C). The 220 samples were ran in cohorts of 1-89, 90-178 and 179-220. Three clusters can be seen and classified based on the fluorescence values of the HVC and LVC check samples. N=220 seeds

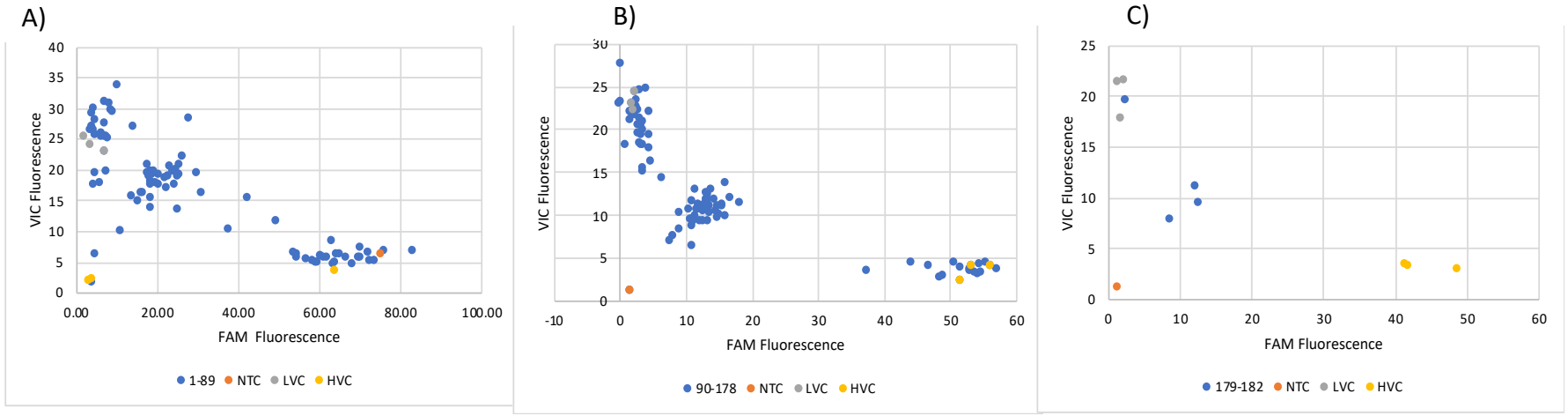

Figure 6.2: Cartesian plot for the allelic discrimination of *vc*- by KASP for the F2 population Disco/2 x Gelber. FAM fluorescence and VIC fluorescence are on the x and y axis respectively. This data was recorded at time point 6(A) and 5 (B & C). The 182 samples were ran in cohorts of 1-89, 90-178 and 179-182. Three clusters can be seen and classified based on the fluorescence values of the HVC and LVC check samples. N=182 seeds

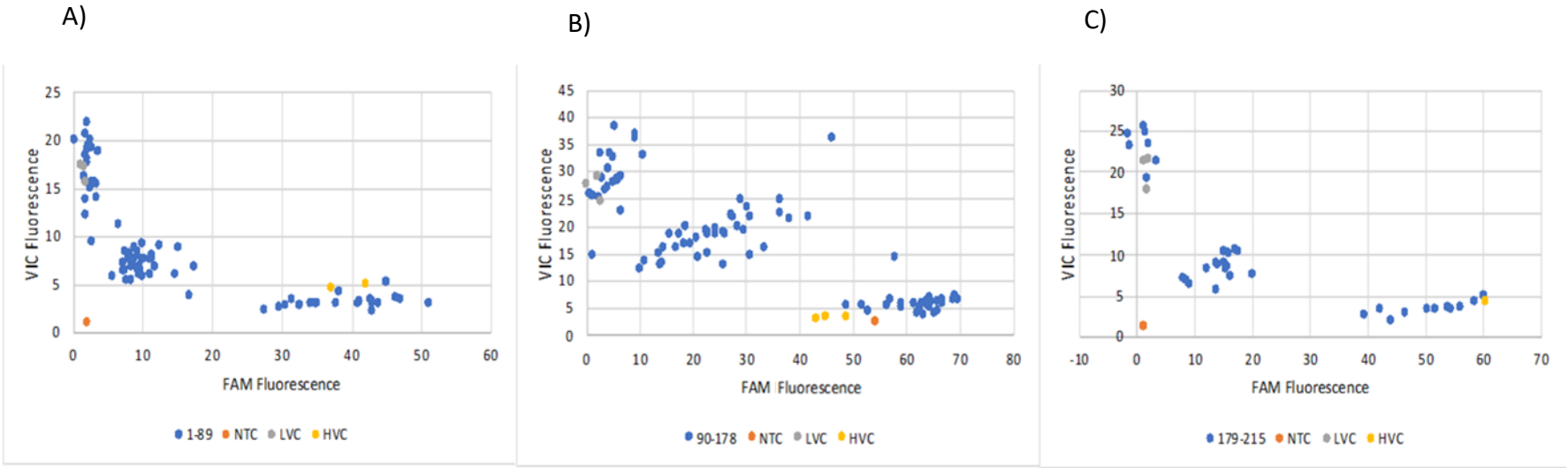

Figure 6.3: Cartesian plot for the allelic discrimination of *vc*- by KASP for the F2 population Disco/2 x P47-1. FAM fluorescence and VIC fluorescence are on the x and y axis respectively. This data was recorded at time point 5 (A & C) and 6 (B). The 182 samples were ran in cohorts of 1-89, 90-178 and 179-215.Three clusters can be seen and classified based on the fluorescence values of the HVC and LVC check samples. N=215 seeds

6.4.2 Mendelian Segregation

Table 6.1: Chi-square results for 3:1 segregation of the *vc-* gene within the phenotypic classes of 3 populations segregating for dwarfism, flower colour and wing flower spot with 1 degree of freedom. Data from cotyledon tissue genotyped by KASP assay for the presence of the *vc-* gene.

Cross	Number of plants				
F2	HVC/HET	LVC	Expected Ratio	X^2	P
Rinrei x Disco/2					
Dwarf	6	1	3:1	0.429	0.513*
Normal	21	9	3:1	0.400	0.527*
P47-1 x Disco/2					
Brown Spot	24	5	3:1	1.111	0.295*
White	7	2	3:1	0.037	0.847*
Pink	3	2	3:1	0.600	0.439*
Brown	2	1	3:1	0.111	0.739*
Red	0	0	3:1	n.a	n.a
Disco/2 x Gelber					
Brown Spot	20	11	3:1	1.817	0.178*
Yellow Spot	8	3	3:1	0.030	0.862*
White	9	4	3:1	0.231	0.631*

HVC=Homozygous for high vicine-convicine
HET=Heterozygous for high vicine-convicine and expressing high vicine-convicine
LVC=Homozygous for low vicine-convicine
*=p-value >0.05 , null hypothesis is accepted (observed ratio did not significantly vary from expected)

Three F2 populations were examined to determine if the VC marker was linked with any of the traits of interest. A chi-square analysis was done for each phenotype within each population to determine if any of the phenotypes are linked to LVC or HVC genotypes (Table 6.1). All the populations and phenotypes within the categories fit a 3:1 Mendelian ratio for the single recessive *vc*- marker, indicating there is no linkage. Hypothesis v was therefore accepted, indicating no linkage between the vicine-convicine gene and the traits of interest.

6.5 Discussion

Vicine-convicine content is a very important quality characteristic for faba bean regardless of end use. The increase in globalization increases risk for individuals with the G6PD enzyme deficiency who are potentially unaware due to the change in culture. Faba bean as an edible vegetable is very uncommon in North America; therefore, second or first generation Canadians may have never been exposed to the dangers of VC, unlike their parents. In order to market faba bean successfully as a vegetable or ornamental crop in North America, it is crucial that these antinutritional compounds are greatly reduced. It is therefore imperative to know if the VC marker is linked with any of the vegetable or ornamental traits. Figure 6.1-6.3 show the analysis of 3 populations segregating for LVC. The allelic discrimination is easily discernable through the three clusters of data points, indicating the allelic form of the LVC marker. Another observation from these images is the slight shift towards the VIC fluorescence in the heterozygous data points. This is likely an indicator that there is more than one gene impacting the LVC genotype (Unpublished data, 2019). Since the LVC marker used in this KASP reaction is known to be a single recessive gene, the 3:1 ratio was used to compare all phenotypes to determine if any of the phenotypes did not fit the expected ratio, which would indicate potential linkage. Table 6.1 shows the phenotypes of interest present in the 3 genotyped populations. All the phenotypes and their VC genotype fit the expected 3:1 ratio ($p>0.05$). Although this table describes no linkage, there may be other factors influencing these data. The main factor reducing the reliability of this data is population size. As shown in Figures 6.1-6.3, the populations analyzed have around 200 individuals, in contrast to Table 6.1, where the populations are reduced to 50 or less. According to Muller's formula, a few of these populations are not large enough for 95% accuracy (Table 3.3). The population loss is due to the field conditions in which these plants were grown. The specific area of the field had a multitude of agronomic problems,

including soil compaction, strong weed pressure and cutworms (*Agrotis orthogonia*). The source of LVC was a low tannin variety, which is known to reduce emergence and seedling vigour (Kantar et al., 1996). Additionally, all the seeds had a portion of the seed coat removed in order to collect DNA from the cotyledon. Since the seed coat plays an important role in defense against both biotic and abiotic stress (Mohamed –Yasseen et al., 1994) the scarification could have factored in the poor emergence. The consequence of the poor emergence is that some of the phenotypes were not expressed.

6.6 Conclusion

The LVC marker is not genetically linked to the Rinrei type dwarfism nor to flower colour. Although there was a drastic reduction in population size between genotyping and phenotyping, the chi-square results gave a p-value > 0.05. A 3:1 model for the *vc*- gene was expressed within each phenotypic class. Hypothesis v is accepted, although this experiment should be replicated to ensure the population size did not reduce the efficacy of the results. However, these are encouraging results for the use of faba bean in the vegetable and ornamental industries.

CHAPTER 7- GENERAL DISCUSSION

7.1 Discussion

Faba bean is a very diverse species with many agronomic, food and feed uses. Although research has lagged behind in both field and vegetable markets in comparison to other legume species, research in field type faba bean has seen a recent increase. However, few significant contributions have been made to increase use of faba bean in the vegetable industry in North America. The soil health applications for large and small scale agriculture are significant based on the robust nitrogen fixation abilities of faba bean. The high protein content makes it an excellent crop for the plan-based protein market. With the ability to manipulate flower colour, plant architecture and reduce antinutritional compounds, the breeding potential is endless for this diverse species.

Faba bean flowers can be found in many colours, although few are available in the current market. Aside from their ability to produce large clusters of beautiful flowers, faba bean flowers are also edible. Consuming flowers is not a new concept, in China and Japan people have been eating flowers for thousands of years (Yang and Walters, 1992). Flowers have unique nutritional aspects that are different from the fruit they set. Non-toxic flowers have a high nutritional value because of their minerals and antioxidants, and they have higher mineral content than most fruit or vegetable species (Rop et al., 2012).

Plant architecture is an important breeding component for faba bean. The modification of plant architecture is known to have a significant impact on crop adaptation and yield (Busov et al., 2008; Wang and Li, 2008). The huge amount of diversity over many architectural traits of faba bean facilitates breeding for multiple uses, as well as optimizing plant yield for the current markets. For yield optimization in a similar species (example: soybean), breeders target tall plants with many branches (Sun et al., 2019). Plant architecture is also being studied in *Brassica napu*s to determine how the architecture in this species impacts yield. Although yield was not a major focus of this study, it can be assumed that there are yield variations within the multiple plant structures studied. Plant architecture is a common focus for large scale agriculture, but it is also a crucial component for the horticulture industry. Another notable observation is that a substitute for black mulch should be used to avoid heat cankers on delicate transplants in future experiments.

The esthetic value of vegetable crop plants is often overlooked since the end use of the plant is clearly defined as functional. However, creating vegetable crops that are beautiful can lead to a more sustainable ornamental and horticulture industry. The edible ornamental market does exist; however, there seems to be a constant compromise between taste and esthetics. Faba bean is an excellent species to show that these are not mutually exclusive characteristics. The flower colour diversity and unique dwarfism characteristics in this species make endless possibilities for the edible ornamental and horticultural markets.

Although yield is an important factor for all crops, the antinutritional compounds in faba bean pose a greater challenge. In order for faba bean to successfully cross into the protein fractionation market and the vegetable market, vicine and convince need to be bred out of the cultivated material. Although these compounds only negatively affect a small portion of the total human population with the G6PD enzyme deficiency, it is still the most common enzyme defect in the human race (Cappellini and Fiorelli, 2008). Therefore, VC must be significantly reduced for faba bean to really be successful. This is particularly relevant for the protein market, since plant based proteins are often used as an ingredient in other products without being clearly labeled. Since the development of the LVC marker, this challenge has been significantly reduced. For the vegetable and ornamental markets, developing LVC plants will boost production, since it will be safe for the entire population. The reduction of these antinutrionals was the last barrier holding faba bean back from its diverse markets and end uses. The future of this species has never been brighter.

7.2 General Conclusions

Faba bean (*Vicia faba*) has a huge amount of potential for diversifying the horticulture industry in multiple sectors. The branching variation coupled with flower colour diversity make it an excellent potential cut flower. The dwarfism characteristics could be used in nursery and bedding plant systems in a range of colours. Lastly, the vegetable type faba bean market is extremely limited for home garden use and field scale vegetable production. The current publicly available types are very old and lack phenotypic variability.

7.3 Future Work

The future work to be done based on the conclusions and observations in this study are as follows. In regard to flower colour, larger population sizes of F2s should be developed to fully and statistically test the trihybrid nature of some of these crosses. Additionally, more backcross studies should be done for crosses where epistasis was observed. To determine the genotype of the intermediate heterozygous flower colours for the red flower genes, a KASP marker could be developed to determine the allelic state of the intermediate flower colour phenotypes. These markers could also be used alongside the LVC marker to rapidly screen for LVC genotypes with the unique flower colours.

Further work should also be done with the Rinrei type dwarfism/slow growth gene. Since plants expressing this gene still reach normal height after a prolonged period, a temperature controlled experiment should be conducted. Rinrei is derived from a winter bean; therefore, its growth cycle could require a vernalisation period. By giving these Rinrei-type plants a cold stress, they may correct for the slow growth. This would indicate the gene controlling this slow growth is linked to the winter bean background of Rinrei, rather than a discovery from the mutagenesis work that created it. The last area where further work would be beneficial is in polyphenols. Polyphenols are the key components influencing flower colour, and could give a better understanding of why there are various hues found within the same colour classes. Appendix D shows that such data was collected and partially analyzed to determine if there are any unique polyphenols for certain flower colours. A genotype by environment analysis should be done to determine how genotype influences the flower colour, and these data could be further investigated to determine significance.

Analysis for other important ornamental traits should also be studied. Important characteristics including the lifespan of the flowers and floriferousness. These are essential characteristics for the floriculture and nursery plant markets. Many of the beautiful flower colours examined in this study are in a homozygous and genetically stable state. These colours would be the first candidates for future analysis of market potential.

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APPENDICIES

Appendix A: Flower Colour Summary

Table A.1: Chi square analysis for P47 x Aurora and reciprocal for 9:3:4 and 12:3:1, recessive and dominant epistasis, respectively.

Generation	Number of plants observed						
F2	white	pink	brown	red	Expected Ratio	X^2	P
P47 x Aurora	61	15	0	14	9:3:4	5.116	0.077
Aurora x P47	85	24	0	7	12:3:1	0.287	0.866

Table A.2: Colour comparison for 12 F2 populations for the standard petal.

Generation		Phenotypes Present							
F2	Tissue	White	Pink	Brown	Red	Yellow Spot	Pink/Yellow	Solid Yellow	Red/Yellow
Aurora x P47-1	Standard	Y	Y	N	Y	N	N	N	N
P47-1 x Aurora	Standard	Y	Y	N	Y	N	N	N	N
P47-1 x Disco/2	Standard	Y	Y	Y	Y	N	N	N	N
Disco/2 x P47-1	Standard	Y	Y	Y	Y	N	N	N	N
Gelber x P47-1	Standard	Y	Y	Y	Y	Y	Y	Y	Y
P47 -1 x Gelber	Standard	Y	Y	Y	Y	Y	Y	Y	Y
CDC Snowdrop x P47-1	Standard	Y	Y	N	Y	N	N	N	N
P47-1 x CDC Snowdrop	Standard	Y	Y	N	Y	N	N	N	N
IG 114476 x P47-1	Standard	Y	Y	Y	Y	N	N	N	N
Rinrei x P47-1	Standard	Y	Y	Y	Y	N	N	N	N
P47-1 x Rinrei	Standard	Y	Y	Y	Y	N	N	N	N
P47-1 x NV153	Standard	Y	Y	Y	Y	N	N	N	N

Table A.3: Colour comparison for 12 F2 populations for the wing petal.

Generation		Phenotypes Present							
F2	Tissue	Brown Spot	White	Pink/Brown	Brown	Red	Yellow Spot	Solid Yellow	Red/Yellow
Aurora x P47-1	Wing	Y	N	Y	Y	Y	N	N	N
P47-1 x Aurora	Wing	Y	N	Y	Y	Y	N	N	N
P47-1 x Disco/2	Wing	Y	Y	Y	Y	Y	N	N	N
Disco/2 x P47-1	Wing	Y	Y	Y	Y	Y	N	N	N
Gelber x P47-1	Wing	Y	N	Y	Y	Y	Y	Y	Y
P47 -1 x Gelber	Wing	Y	N	Y	Y	Y	Y	Y	Y
CDC Snowdrop x P47-1	Wing	Y	Y	Y	N	Y	N	N	N
P47-1 x CDC Snowdrop	Wing	Y	Y	Y	N	Y	N	N	N
IG 114476 x P47-1	Wing	Y	N	Y	Y	Y	N	N	N
Rinrei x P47-1	Wing	Y	N	Y	Y	Y	N	N	N
P47-1 x Rinrei	Wing	Y	N	Y	Y	Y	N	N	N
P47-1 x NV153	Wing	Y	N	Y	Y	Y	N	N	N

Appendix B: Mean, Variation, Standard Deviation and range for Height and Average Internode length of all populations in their respective locations.

Table B.1: The mean, variation, standard deviation and range for the height measurement for both parent lines and the segregating F2 population Rinrei x NV153 in two locations.

Height Data

Location	Cross	Mean	Variation	Std Dev	Range
Field	Rinrei	41.00	24.66	6.08	37.00-48.00
Phytotron	Rinrei	46.75	26.44	5.50	39.00-53.00
Field	NV153	30.57	4.82	2.37	27.00-34.00
Phytotron	NV153	19.71	0.78	0.95	19.00-21.00
Field	Rinrei x NV153	48.60	301.00	17.30	11.00-90.00
Phytotron	Rinrei x NV153	51.67	591.78	24.33	14.00-105.00

Table B.2: The mean, variation, standard deviation and range for the average internode length for both parent lines and the segregating F2 population Rinrei x NV153 in two locations.

Avg. Internode

Location	Cross	Mean	Variation	Std Dev	Range
Field	Rinrei	1.62	0.01	0.13	1.48-1.73
Phytotron	Rinrei	1.40	0.01	0.13	1.28-1.68
Field	NV153	1.02	0.01	0.09	0.94-1.15
Phytotron	NV153	0.62	0.01	0.07	0.51-0.72
Field	Rinrei x NV153	1.77	0.38	0.61	0.44-3.29
Phytotron	Rinrei x NV153	1.72	0.59	0.77	0.45-3.75

Table B.3: The mean, variation, standard deviation and range for plant height of both parental lines and the segregating F2 population Aurora x NV153 in two locations.

Height Data

Location	Cross	Mean	Variation	Std Dev	Range
Field	Aurora	81.00	67.60	9.19	71.00-96.00
Phytotron	Aurora	135.00	193.50	16.06	112.00-147.00
Field	NV153	30.57	4.82	2.37	27.00-34.00
Phytotron	NV153	19.71	0.78	0.95	19.00-21.00
Field	Aurora x NV153	56.14	241.60	15.54	20.00-99.00
Phytotron	Aurora x NV153	85.55	940.68	30.67	31.00-146.00

Table B.4: The mean, variation, standard deviation and range for the average internode length from field data of both parent lines and the segregating F2 population Aurora x NV153 in two locations.

Avg. Internode

Location	Cross	Mean	Variation	Std Dev	Range
Field	Aurora	2.79	0.24	0.55	2.29-3.69
Phytotron	Aurora	3.39	0.07	0.30	3.06-3.73
Field	NV153	1.03	0.01	0.09	0.94-1.15
Phytotron	NV153	0.62	0.01	0.07	0.51-0.72
Field	Aurora x NV153	2.20	0.35	0.59	0.97-4.30
Phytotron	Aurora x NV153	2.39	0.65	0.81	0.84-3.76

Table B.5: The mean, variation, standard deviation and range for plant height of parental lines and segregating F2 population for the cross IG114476 x NV153 in two locations.

Height Data

Location	Cross	Mean	Variation	Std Dev	Range
Field	IG1114476	26.57	5.39	2.51	23.00-30.00
Phytotron	IG1114476	33.17	55.81	8.08	23.00-45.00
Field	NV153	30.57	4.82	2.37	27.00-34.00
Phytotron	NV153	19.71	0.78	0.95	19.00-21.00
Field	IG114476 x NV153	30.07	119.08	10.91	9.00-64.00
Phytotron	IG114476 x NV153	45.72	331.38	18.20	12.00-94.00

Table B.6: The mean, variation, standard deviation and range for the average internode length from field data of both parent lines and the segregating F2 population IG114476 x NV153 in two locations.

Avg. Internode

Location	Cross	Mean	Variation	Std Dev	Range
Field	IG114476	1.52	0.08	0.30	0.91-1.86
Phytotron	IG114476	1.75	0.01	0.11	1.53-1.86
Field	NV153	1.03	0.01	0.09	0.94-1.15
Phytotron	NV153	0.61	0.01	0.07	0.51-0.72
Field	IG114476 x NV153	1.33	0.19	0.44	0.56-2.56
Phytotron	IG114476 x NV153	1.86	0.46	0.68	0.58-3.03

Table B.7: The mean, variation, standard deviation and range for the height measurement from both parent lines and the segregating F2 populations Rinrei x Aurora & P47-1 x Rinrei.

Height Data

Location	Cross	Mean	Variation	Std Dev	Range
Field	Rinrei	41.00	24.67	6.08	37.00-48.00
Phytotron	Rinrei	46.75	26.44	5.50	39.00-53.00
Field	Aurora	81.00	67.60	9.19	71.00-96.00
Phytotron	Aurora	135.00	193.50	16.06	112.00-147.00
Phytotron	P47-1	30.57	4.82	2.37	27.00-34.00
Field	Rinrei x Aurora	72.60	413.31	20.33	29.00-117.00
Phytotron	P47-1 x Rinrei	80.88	298.38	17.27	49.00-110.00

Table B.8: The mean, variation, standard deviation and range for the average internode length from field data of both parent lines and the segregating F2 populations Rinrei x Aurora & P47-1 x Rinrei.

Avg. Internode

Location	Cross	Mean	Variation	Std Dev	Range
Field	Rinrei	1.62	0.01	0.13	1.48-1.73
Phytotron	Rinrei	1.40	0.01	0.13	1.28-1.68
Field	Aurora	2.79	0.24	0.55	2.29-3.69
Phytotron	Aurora	3.39	0.07	0.30	3.06-3.73
Phytotron	P47-1	2.18	0.00	0.09	2.10-2.29
Field	Rinrei x Aurora	2.50	0.29	0.53	1.19-4.00
Phytotron	P47-1 x Rinrei	2.75	0.57	0.76	0.99-3.67

Table B.9: The mean, variation, standard deviation and range for the height measurement from both parent lines and the segregating F2 population Rinrei x IG114476.

Height(cm)	Mean	Variation	Std Dev	Range
Rinrei	41.000	24.667	6.082	37.00-48.00
IG114476	26.571	5.388	2.507	23.00-30.00
Rinrei x IG114476	33.864	61.423	7.837	20.00-52.00

Table B.10: The mean, variation, standard deviation and range for the average internode length from both parent lines and the segregating F2 population Rinrei x IG114476.

Avg. Internode	Mean	Variation	Std Dev	Range
Rinrei	1.621	0.011	0.127	1.48-1.72
IG114476	1.516	0.078	0.301	0.91-1.86
Rinrei x IG114476	1.606	0.086	0.293	1.14-2.69

Table B.11: The mean, variation, standard deviation and range for the height measurement from both parent lines and the segregating F2 population IG114476 x Rinrei.

Height(cm)	Mean	Variation	Std Dev	Range
IG114476	26.571	5.388	2.507	23.00-30.00
Rinrei	41.000	24.667	6.082	37.00-48.00
IG114476 x Rinrei	35.993	69.783	8.354	17.00-70.00

Table B.12: The mean, variation, standard deviation and range for the average internode length from both parent lines and the segregating F2 population IG114476 x Rinrei.

Avg. Internode	Mean	Variation	Std Dev	Range
IG114476	1.516	0.078	0.301	0.91-1.86
Rinrei	1.621	0.011	0.127	1.48-1.72
IG114476 xRinrei	1.746	0.096	0.311	0.90-3.11

Table B.13: The mean, variation, standard deviation and range for the number of branches for both parent lines and the segregating F2 population IG114476 x Aurora in two locations.

Branching

Location	Cross	Mean	Variation	Std Dev	Range
Field	IG114476	4.60	1.84	1.52	3.00-8.00
Phytotron	IG114476	4.50	0.92	0.84	3.00-6.00
Field	Auora	5.43	2.82	1.81	3.00-6.00
Phytotron	Auora	1.50	0.25	0.58	1.00-2.00
Field	IG114476 x Aurora	6.04	10.47	3.24	1.00-21.00
Phytotron	IG114476 x Aurora	3.14	0.75	0.87	1.00-5.00

Table B.14: The mean, variation, standard deviation and range for the number of branches for both parent lines and the segregating F2 population Aurora x IG114476 in two locations.

Branching

Location	Cross	Mean	Variation	Std Dev	Range
Field	IG114476	4.60	1.84	1.52	3.00-8.00
Phytotron	IG114476	4.50	0.92	0.84	3.00-6.00
Field	Auora	5.43	2.82	1.81	3.00-6.00
Phytotron	Auora	1.50	0.25	0.58	1.00-2.00
Field	Aurora x IG114476	8.50	17.67	4.20	2.00-24.00
Phytotron	Aurora x IG114476	3.00	0.63	0.79	1.00-5.00

Table B.15: The mean, variation, standard deviation and range for the number of branches for both parent lines and the segregating F2 population IG114476 x NV153 in two locations.

Branching

Location	Cross	Mean	Variation	Std Dev	Range
Field	IG114476	5.43	2.82	1.81	3.00-8.00
Phytotron	IG114476	4.50	0.91	0.84	3.00-6.00
Field	NV153	6.14	0.98	1.07	5.00-8.00
Phytotron	NV153	6.14	0.98	1.07	5.00-8.00
Field	IG114476 x NV153	9.07	15.05	3.68	2.00-26.00
Phytotron	IG114476 x NV153	4.59	2.49	1.58	2.00-10.00

Table B.16: The mean, variation, standard deviation and range for the number of branches for both parent lines and the segregating F2 population IG114476 x Rinrei and its reciprocal Rinrei x IG114476.

Branching					
Location	Cross	Mean	Variation	Std Dev	Range
Field	Rinrei	6.00	0.67	1.00	5.00-7.00
Field	IG114476	5.43	2.82	1.81	3.00-8.00
Field	Rinrei z IG114476	8.52	9.05	3.00	4.00-16.00
Field	IG114476 x Rinrei	6.86	10.92	3.30	1.00-22.00

Appendix C: LGC sample collection & KASP PCR program

Plant sample collection kit

Contents

1. Introduction

The purpose of this document is to explain how to collect and pack leaf tissue samples using the LGC plant sample collection kit (KBS-9370-001). This step-by-step guide will ensure that leaf tissue collection is carried out in the most optimal way to ensure that samples arrive at LGC in the best possible condition.

2. Kit contents and customer requirements

Kit contents

- 1 x 96-well tube storage rack with lid, containing 12 x 8-strip tubes
- 12 x perforated 8-strip caps
- 1 x 50 g desiccant sachet. Please note that this sachet will arrive in a plastic bag. Do not remove it from this bag until plates are being prepared for shipping as this will dramatically reduce its ability to desiccate leaf samples.
- 1 x large labelled sealable bag
- Elastic band
- 1 x leaf cutting tool (where applicable)
- 1 x leaf cutting mat (where applicable)
  Please note: If multiple kits have been requested, only one cutting tool and one cutting mat will be sent to you.
- Access to plants from which leaf tissue is to be collected
- Clean water (to wash cutting tool)
- Suitable box / container for shipping the completed package to LGC
- Information from LGC regarding the number of leaf discs required per sample for your specific project (See Section 5 for more details).

3. Recommendations before you start

- Familiarise yourself with the components of the kit
- Read through the step-by-step guide to ensure that you understand all of the steps
- Ensure that the desiccant sachet is still securely sealed in the plastic bag. Do not open this bag until you are preparing the samples for shipping.
- Prepare a dish of clean water for washing the cutting tool in between sampling of each individual plant.
- Label the 96-well tube storage rack(s) with a unique ID.
- Ensure that you have discussed your project with LGC, and that you have subsequently been advised regarding the appropriate number of leaf discs that are required per individual plant (see Section 5 for more details).

4. Overview of the procedure

- Cut the leaf discs from the relevant leaf material
- Add the leaf discs to the tube storage rack (supplied) and seal the tubes
- Place the desiccant sachet on top of the sealed tubes, and fix the rack lid in place
- Place the prepared tube storage rack inside a plastic bag (supplied) and seal the bag
- Package suitably for shipping, ensuring that an appropriate description for customs is included

5. Determining the appropriate number of leaf discs per sample

Before you receive this kit from LGC, you will be required to discuss your project with a member of our team. Based on information that you provide, LGC can advise on the number of leaf discs required per individual sample for your specific project.

It is important to determine the appropriate number of leaf discs that are required before commencing with leaf disc collection. The number of leaf discs required per sample will differ depending on the genome size of your study organism, the number of SNPs that require genotyping for these samples, and the nature of the leaf tissue itself. The larger the genome size, the greater the number of leaf discs that will be required. Likewise, the greater the number of SNPs that are to be subsequently analysed, the greater the number of leaf discs that will be required to ensure that there is sufficient DNA available.

Figure C.1: LGC sample collection kit page 1

6. Step-by-step guide to collecting leaf tissue samples

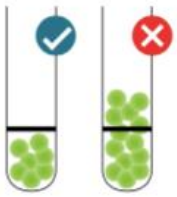
1. Remove the cap from the leaf cutting tool.

2. Place the leaf to be sampled on the leaf cutting mat. Please note: the leaf does not have to be detached from the plant.

3. Hold the cutting tool vertically above the leaf and then push the tool into the leaf tissue. Twist thetool (i.e. turn clockwise) as it is being pushed into the leaf tissue to cut and pick the leaf disc up in the cutting tool.

4. Insert the end of the cutting tool into the first well of the 96-well tube storage rack and depress the plunger to dispense the leaf disc. Please note: the tubes are in strips of 8 and can be lifted out of the rack to aid dispensing of the leaf disc.

5. Repeat steps 3 and 4 until you have collected the appropriate number of leaf discs for the first leaf sample *(NB. LGC will advise you regarding the number of leaf discs required, see Section 5).*



Please do not fill the tube above the black line as indicated in the picture.

6. Once sufficient leaf discs have been sampled from the first plant, the cutting tool needs to be washed. Place the end of the cutting tool into a container of clean water and depress the plunger 5-10 times.
7. After washing the cutting tool, flick / shake the tool until it is completely dry.
8. Repeat steps 1 – 7 for all of the remaining plants that require sampling. Ensure that the cutting tool is washed (steps 6 and 7) in between sampling of each individual plant.
9. Ensure that the 96-well tube storage rack containing collected leaf discs is labelled appropriately.

7. Step-by-step guide to packing leaf tissue samples for shipping

1. Once the 96-well storage rack is full (or all required plants have been sampled), seal each strip of tubes within the rack using the perforated strip caps provided. Press each cap firmly into place to ensure that the tubes are securely sealed.

2. Remove the desiccant sachet from the sealed plastic bag (NB. this must not be removed from the bag until plates are being prepared for shipping).

3. Place the desiccant sachet directly on top of the strip capsealed tubes.

4. Replace the plastic lid on top of the 96-well storage rack, ensuring that the desiccant sachet is situated beneath the plastic lid. Please be aware that the plastic lid will not fit tightly onto the base of the rack due to the presence of the desiccant sachet.

5. Secure the lid in place using the elastic band provided.

6. Place the sealed rack into the large labelled sealable bag provided. Force excess air out of the bag and seal the bag tightly.

7. Place the sealed bag into the original plant kit box.
8. Place the completed leaf sampling kit(s) into a suitable container for shipping to LGC.
9. Prepare a description of the package contents for customs.
10. Send the completed package to the appropriate LGC site, as advised by your project manager.

11. Complete an LGC plate map file (available from our website) and send this to your project manager at LGC. Your project can then be set up in our database in advance of receiving your leaf tissue sample plates.

Shipping addresses:

USA
LGC Genomics, 100 Cummings Center, Suite 420H
Beverly, MA 01915, USA
Tel: + 1 978 232 9430 Email: sales.us@lgcgroup.com

UK
LGC Genomics, Unit 27 Trident Industrial Estate
Pindar Road, Hoddesdon, Herts EN11 0DE, UK
Tel: +44 (0)1992 470 757 Email: orders.uk@lgcgroup.com

Germany
LGC Genomics, Ostendstraße 25/Haus 8
12459 Berlin, Germany
Tel: +49 (0)30 5304 2200 Email: info.de@lgcgroup.com

Figure C.2: LGC sample collection kit page 2

PCR Program for KASP Assays

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21°.....2 min – followed by fluorescence reading (PRE-PCR ReaD)

95°.....15 min

10 cycles:

/ 94°..... 20 sec

\ 65° (Δ-0.8°/cyl). 1 min

32 cycles:

/ 94°..... 20 sec

\ 57°..... 1 min

21°.....2 min – followed by fluorescence reading (POST-PCR Read 1)

****

16 cycles:

/ 95°.....20 sec

\ 57°.....1 min

(After every 4 cycles)

21°.....2 min – followed by fluorescence reading (POST-PCR read 2-5)

****

Figure C.3: The PCR program used for the KASP assay of *vc*-. Developed by Rob Stonehouse, (2018) at the University of Saskatchewan.

Appendix D: Mass Spectrometry: Polyphenol Profiles of flower colour

Material and method

Plant materials

The flower tissue for this experiment was collected from parent genotypes as well as segregating F2 plants. These flowers were harvested on February 8th 2018 from the phytotron F2 location referred to in section 4.1.2. The flowers were manually dissected according to petal type. The standard petal was removed and kept separate from the wing and keel petals. The flower tissue samples were placed in two 5ml tubes based on petal tissue type and loosely sealed with aluminum foil. The 50ml tubes were then placed in liquid nitrogen and frozen. Samples were collected for each phenotype within 10 F2 populations and the parent genotypes. The same phenotypes were pooled to achieve the necessary volume. Once all the samples were collected, these samples were placed in -80°C.

Sample preparation

The samples were removed from the freezer and freeze dried in a 6 Liter FreeZone (Labconco, Kansas City USA) for 48 hours. Once the flowers were dried, they were sealed and stored at room temperature. On January 17th 2018, the samples were freeze dried again for 24 hours and then ground. The samples were placed into 5ml tubes with two yttria stabilized zirconia(YSZ) grinding media 6.6mm beads (Inframat Advanced Materials, Manchester USA) and ground for 2 minutes and 30 seconds at 1300rmp on a 2010 Geno/Grinder (SPEX® SamplePrep, NJ USA). 15mg of each sample was weighed on a H51 Sartorius handy Laboratory Scale (Sartorius Goettingen, Germany) and placed in 1.5ml micro-tubes. The extraction methods were adapted from the seed coat method of Mirali et al (2014) to analyze the flower tissue. To

extract the supernatant, 1ml of acetone: water (70:30 v/v) containing internal standards (IS) was added to each sample. The IS were added to adjust for matrix effects and ensure accurate quantification. The samples were then shaken for 1 hour on an Eppendorf ThermoMixer (Eppendorf, New York USA) mixer at 1400rpm and 23°C. Next, the samples were centrifuged on Sorvall Legend Micro 17 Centrifuge (Thermo Fisher Scientific, Waltham USA) at 13000rpm for 15 minutes. The supernatant was then collected (500µL) and centrifuged again at 13000rpm and 15 minutes. The supernatant was collected for a second time (200µL) and placed in 1.5ml Eppendorf tubes and dried down. After approx. 3 hours, the samples were dry and reconstituted with 200µL 90:10 MiliQ water: methanol.

Liquid chromatography-diode array detector and a mass spectrometer (LC-DAD-MS)

In this experiment a LC-DAD-MS method was used to identify and quantify the polyphenols of interest. Reverse-phase LC separation was done before the eluent passed through a Vanquish UHPLC (including DAD) and then a TSQ Altis triple quadrupole mass spectrometer (Thermo Fisher Scientific, Waltham USA). The column used for chromatographic separation was an Agilent poroshell 120 PFP, (2.1 x 100 mm) with 2.7 µm particle size (Agilent, Santa Clara, USA). For the LC, the mobile phases used were 1% formic acid in water (solvent A) and Acetonitrile: water: Formic acid 90:9:1, v/v/v(solvent B). The mobile phases in Table 29 were adjusted from Mirali et al. (2016) to improve detectability of phenolic acids as recommended by Fatma Elessawy (unpublished manuscript, 2019). The column pressure was 4500-4900 psi and the column temperature was 25°C. The mobile phase was ran at a flow rate of 0.35mL/min with an injection volume of 5µL. A 3 minute purge was done between each compound to clean the lines.

Table D.1: Mobile phase solvent ratio used during the LC-(DAD)-MS, where A is water: formic acid (99:1,v/v) and B is water: acetonitrile: formic acid (9:90:1,v/v/v)

Time (min)	0	1	21	24	24.1	26	26.1	30
%A	99	99	59	40	20	20	99	99
%B	1	1	41	60	80	80	1	1

The Atlis triple quadruple mass spectrometer received the samples after they went through the LC and ionization. The ionization method used was electro spray. The samples were ran on selected reaction monitoring (SRM) mode and in full scan mode. The SRM mode utilized the standards for quantification. The full scan mode was used in combination with the DAD to investigate compounds that were not identified by the standards. Table 30 shows the complete list of polyphenols that were measured in this experiment and their relevant biochemical properties. There were 98 compounds in 13 phenolic categories (anthocyanins, chalcones, flavonols, flavones, flavan-3-ols, flavanones, flavanonols, dihydroflavonols, stilbenes, hydroxybenzoic acids, hydroxycinnamic acids, isoflavones and proanthocyanidins) tested for in this experiment. Two of the compounds tested (kaempferol-di-rutinoside and catechin-glucoside) did not have commercially available internal standards but were incorporated because they are known polyphenols found in other pulse crops (Mirali et al., 2016).

Table D.2: Polyphenol compounds used as standards for SRM mass spectrometry grouped by phenolic class with their biochemically relevant properties and supplier

Peak #	Compound name	Supplier (source)	Polarity	Precursor ion (*m/z*)	Product ion (*m/z*)	CE (eV)	S-lens voltage (V)	R_t (min)
Anthocyanins								
1	Callistephin	Extrasynthese, France	NEG	431	269	18	116	6.51
2	Malvidin-3-*O*-glucoside (oenin)	Extrasynthese, France	NEG	491	313	35	143	7.69
3	Pelargonin	Extrasynthese, France	NEG	593	431	16	193	5.57
4	Peonidin-3,5-di-*O*-glucoside	Extrasynthese, France	NEG	623	299	30	249	6.16
5	Peonidin-3-*O*-glucoside	Extrasynthese, France	NEG	461	299	18	130	7.14
6	Cyanidin-3,5-di-*O*-glucoside	Extrasynthese, France	NEG	609	447	19	143	5.01
7	Cyanidin-3-*O*-rhamnoside	Extrasynthese, France	NEG	431	285	20	125	7.30
8	Delphinidin-3,5-di-*O*-glucoside	Extrasynthese, France	NEG	625	299	44	175	4.32
9	Delphinidin-3-*O*-rhamnoside	Extrasynthese, France	NEG	447	300	25	125	6.54
10	Delphinidin-3-β-D-Glucoside	Santa Cruz Biotech, US	NEG	463	300	26	120	5.54
11	Kuromanin	Extrasynthese, France	NEG	447	284	25	124	6.06
12	Malvidin-3,5-di-*O*-glucoside	Extrasynthese, France	NEG	653	329	30	225	6.67
13	Malvidin-3-*O*-galactoside	Extrasynthese, France	NEG	491	313	35	143	7.32
Chalcones								
14	Phloretin	Sigma Aldrich, US	NEG	273	167	16	82	18.94
15	Xanthohumol	Extrasynthese, France	POS	355	179	24	60	25.34
Hydroxycoumarins								
16	4-Hydroxy-6-methylcoumarin	Sigma Aldrich, US	NEG	175	131	19	74	14.68
Flavonols								

17	Kaempferol-3-*O*-rhamnoside	Sigma Aldrich, US	NEG	431	285	20	125	14.45
18	Fisetin	Extrasynthese, France	NEG	285	135	21	100	14.67
19	Isorhamnetin	Sigma Aldrich, US	NEG	315	300	22	108	19.05
20	Kaempferol	Sigma Aldrich, US	NEG	285	187	30	129	18.83
21	Kaempferol 3-*O*-robinoside-7-*O*-rhamnoside (robinin)	Sigma Aldrich, US	NEG	739	593	29	153	10.91
22	Kaempferol 3-*O*-rutinoside-4'-glucoside	Sigma Aldrich, US	NEG	755	593	22	208	10.40
23	Kaempferol-3-*O*-D-galactoside	Sigma Aldrich, US	NEG	447	255	39	122	12.73
24	Kaempferol-3-*O*-glucoside	Extrasynthese, France	NEG	447	285	25	130	13.09
25	Kaempferol-3-*O*-rutinoside	Extrasynthese, France	NEG	593	285	35	180	12.69
26	Kaempferol-7-*O*-glucoside	Extrasynthese, France	NEG	447	285	25	130	13.46
27	Kaempferol-7-*O*-neohesperidoside	Extrasynthese, France	NEG	593	285	35	180	13.28
28	Kaempferol dirutinoside	NA	NEG	901	755	30	150	9.15
29	Myricetin	Sigma Aldrich, US	NEG	317	151	24	104	14.73
30	Myricetin-3-*O*-rhamnoside	Extrasynthese, France	NEG	463	316	26	127	11.97
31	Quercetin	Extrasynthese, France	NEG	301	151	21	93	16.90
32	Quercetin-3,4'-di-*O*-glucoside	Extrasynthese, France	NEG	625	301	32	137	10.59
33	Quercetin-3-*O*-galactoside	Extrasynthese, France	NEG	463	300	26	120	12.08
34	Quercetin-3-*O*-glucoside (Isoquercetrin)	Extrasynthese, France	NEG	463	300	26	120	12.19

35	Quercetin-3-*O*-rhamnoside (Quercitrin)	Extrasynthese, France	NEG	447	300	25	125	13.30
36	Quercetin-3-*O*-rutinoside(Rutin)	Extrasynthese, France	NEG	609	300	37	160	11.80
37	Quercetin-4′-*O*-glucoside (Spiraeoside)	Sigma Aldrich, US	NEG	463	301	18	99	14.22
38	Tiliroside	Extrasynthese, France	NEG	593	285	35	180	17.00
39	**Quercetin-d3**	TRC, Canada	NEG	304	151	22	94	16.88
Flavones								
40	5,7-Dimethoxyflavone	Sigma Aldrich, US	POS	283	239	32	101	21.46
41	Apigenin	Extrasynthese, France	NEG	269	117	35	113	18.92
42	Apigenin-7-*O*-glucoside	Extrasynthese, France	NEG	431	268	34	142	13.86
43	Apigenin-7-*O*-neohesperidoside	Extrasynthese, France	NEG	577	269	30	170	13.60
44	Apigenin-7-*O*-rutinoside	Extrasynthese, France	NEG	577	269	30	170	13.37
45	Vitexin	Extrasynthese, France	NEG	431	311	22	117	11.32
46	Chrysin	Extrasynthese, France	POS	255	153	30	161	21.51
47	Diosmetin	Sigma Aldrich, US	NEG	299	284	22	114	19.14
48	Diosmetin-7-*O*-rutinoside	Sigma Aldrich, US	NEG	607	299	26	125	13.69
49	Flavone	Sigma Aldrich, US	POS	223	121	27	119	20.66
50	Luteolin	Extrasynthese, France	NEG	285	133	34	122	17.24
51	Luteolin-3',7-di-*O*-glucoside	Extrasynthese, France	NEG	609	447	22	152	11.44
52	Luteolin-4'-*O*-glucoside	Extrasynthese, France	NEG	447	285	25	130	14.57
53	Luteolin-7-*O*-glucoside	Extrasynthese, France	NEG	447	285	25	130	12.73

54	Luteolin-7-*O*-rutinoside	Sigma Aldrich, US	NEG	593	285	35	180	12.37
55	Luteolin-8'-*C*-glucoside	Extrasynthese, France	NEG	447	327	22	150	10.68
56	Tangeretin	Extrasynthese, France	POS	373	343	27	101	21.93
57	Vitexin-2'-*O*-rhamnoside	Extrasynthese, France	NEG	577	293	36	154	10.96
Flavan-3-ols								
58	(+)-Catechin	Extrasynthese, France	NEG	289	203	19	96	7.40
59	(-)-catechin gallate	Extrasynthese, France	NEG	441	169	20	103	12.50
60	catechin-3-O-glucoside	NA	NEG	451	137	20	95	7.96
61	(-)-Epicatechin	Extrasynthese, France	NEG	289	203	19	96	8.63
62	(-)-Epicatechin gallate	Extrasynthese, France	NEG	441	169	20	103	11.85
63	(-)-Epigallocatechin	Extrasynthese, France	NEG	305	125	22	95	7.12
64	(-)-Epigallocatechin gallate	Extrasynthese, France	NEG	457	169	17	99	10.08
65	(-)-Gallocatechin	Sigma Aldrich, US	NEG	305	125	22	95	5.47
66	**±-Catechin-2,3,4-$^{13}C_3$**	Sigma Aldrich, US	NEG	292	206	19	95	7.40
Flavanones								
67	Eriocitrin	Extrasynthese, France	NEG	595	287	22	138	11.21
68	Eriodictyol	Extrasynthese, France	NEG	287	151	14	81	15.83
69	Hesperetin	Sigma Aldrich, US	NEG	301	164	24	100	18.16
70	Hesperetin-7-*O*-rutinoside	Sigma Aldrich, US	NEG	609	301	25	136	12.92
71	Isosakuranetin	Extrasynthese, France	POS	287	153	23	75	21.66
72	Narigenin-7-*O*-rutinoside	Sigma Aldrich, US	NEG	579	271	23	157	12.32

73	Naringenin	Sigma Aldrich, US	NEG	271	151	18	82	17.72
Flavanonols								
74	Dihydromyricetin	Sigma Aldrich, US	NEG	319	193	10	63	9.31
75	Dihydrokaempferol	Sigma Aldrich, US	NEG	287	125	21	74	12.98
76	Taxifolin (dihydroquercetin)	Sigma Aldrich, US	NEG	303	125	21	70	11.35
Stilbenes								
77	Resveratrol	Sigma Aldrich, US	NEG	227	143	26	92	14.79
78	Resveratrol-3-β-mono-D-glucoside (Polydatin)	Santa Cruz Biotech, US	NEG	389	227	17	110	11.50
79	**Resveratrol-(4-hydroxyphenyl-$^{13}C_6$)**	Sigma Aldrich, US	NEG	233	149	27	110	14.79
Hydroxybenzoic acids								
80	3,4-Dihydroxybenzoic acid	Sigma Aldrich, US	NEG	153	109	14	55	4.23
81	4-amino salicylic acid	Sigma Aldrich, US	NEG	152	108	15	52	4.07
82	4-hydroxybenzoic acid	Sigma Aldrich, US	NEG	137	93	14	51	5.59
83	Gallic acid	Sigma Aldrich, US	NEG	169	125	14	60	2.65
84	Salicin	Sigma Aldrich, US	NEG	285	123	10	90	3.32
85	Syringic acid	Extrasynthese, France	NEG	197	182	14	66	8.04
86	Vanillic acid	Sigma Aldrich, US	NEG	167	108	19	54	6.98
87	Vanillic acid-4-β-D-glucoside	Sigma Aldrich, US	NEG	329	167	10	98	4.35
88	Vanillin	Sigma Aldrich, US	NEG	151	136	13	46	8.11

89	**Vanillin-(ring-$^{13}C_6$)**	Sigma Aldrich, US	NEG	157	142	13	43	8.10
90	**4-hydroxybenzoic acid - $^{13}C_7$**	Sigma Aldrich, US	NEG	144	99	16	52	5.59
Hydroxycinnamic acids								
91	Caffeic acid	Sigma Aldrich, US	NEG	179	135	16	59	7.70
92	Chlorogenic acid	Sigma Aldrich, US	NEG	353	191	17	60	7.87
93	Ferulic acid (trans)	Sigma Aldrich, US	NEG	193	178	13	57	10.46
94	*p*-Coumaric acid (trans)	Sigma Aldrich, US	NEG	163	119	15	53	9.32
95	*trans*-3-hydroxycinnamic acid	Sigma Aldrich, US	NEG	163	119	15	53	10.22
96	**Ferulic acid-d3**	TRC, Canada	NEG	196	134	17	54	10.44
Isoflavones								
97	Genistein	Sigma Aldrich, US	NEG	269	159	29	124	17.96
98	Prunetin	Sigma Aldrich, US	POS	285	242	32	117	22.21
Procyanidins								
99	Procyanidin A2	Sigma Aldrich, US	NEG	575	285	28	160	11.59
100	Procyanidin B1	Sigma Aldrich, US	NEG	577	289	24	120	6.66
101	Procyanidin B2	Sigma Aldrich, US	NEG	577	289	24	120	7.90
102	Procyanidin B3	AdooQ, US	NEG	577	289	24	120	7.13
103	Procyanidin C1	Sigma Aldrich, US	NEG	865	407	40	151	9.37

The SRM peaks were integrated by using Xcalibur 4.0 with Foundation 3.1(Thermo Fisher Scientific, Waltham USA). First the calibration curve for each compound was manually

examined to confirm R^2>0.99 for the standards. Then the peak integrations for each compound were checked to determine if integration software identified the correct peak. The peaks were then exported to concentration values into Excel 2016 and adjusted based on initial sample weight (g) and molecular weight (mg), as in the formula below, to standardize all the samples from nM to µg/g.

$$\mu M = \frac{wt(nM)}{Mol.wt} x \frac{1000}{Vml}$$

$$\mu g = \frac{\mu M \; x \; Mol.wt \; x \; 1ml}{1000}$$

$$\mu g/ml = \frac{nM \; x \; Mol.wt}{1000 \; x \; 1000}$$

$$\mu g/g = \frac{nM \; x \; Mol.wt}{1000 \; x \; 1000 \; x \; sample \; wt(g)} \quad \text{(D.1)}$$

Statistical analysis

My finalized data was received on April 15th2019. Therefore, due to time constraints, only the initial data visualization was done. The data set is very large with multiple samples expressing the same colour. Thus groups were formed based on flower colour.. The five groups were White, White Pattern, Red, Brown Pink and Brown. The variation was tested using a two factor ANOVA, to determine if these groups need to be adjusted. The category ‘White Pattern” had significant variation within the grouping indicating further data partitioning is required.

The next step is to do an ANOVA to determine significant differences for the polyphenol concentrations between the individual samples and polyphenolic classes. Additionally, a GxE model will be developed to see if the genotype has a significant impact on the polyphenol concentrations. Lastly, I will look for unique concentrations or compounds that are colour specific.

Results

Of the 13 polyphenolic classes, only 10 were present in these samples. These classes are: Anthocyanins, Dihydroflavonols, Flavones, Flavonols, Flavan-3-ols, Flavanones, Hydroxybenzoic Acid, Hydroxycinnamic Acid, Procyanidins and Stilbenes. The mean concentrations varied from 0.08 μg/g to 1131.26 μg/g. The three phenolic classes with the highest concentrations in these samples are: Anthocyanins, Flavones and Hydroxybenzoic Acid. The categorical break down of the concentrations of these three major classes are illustrated in Fig D1-D4.

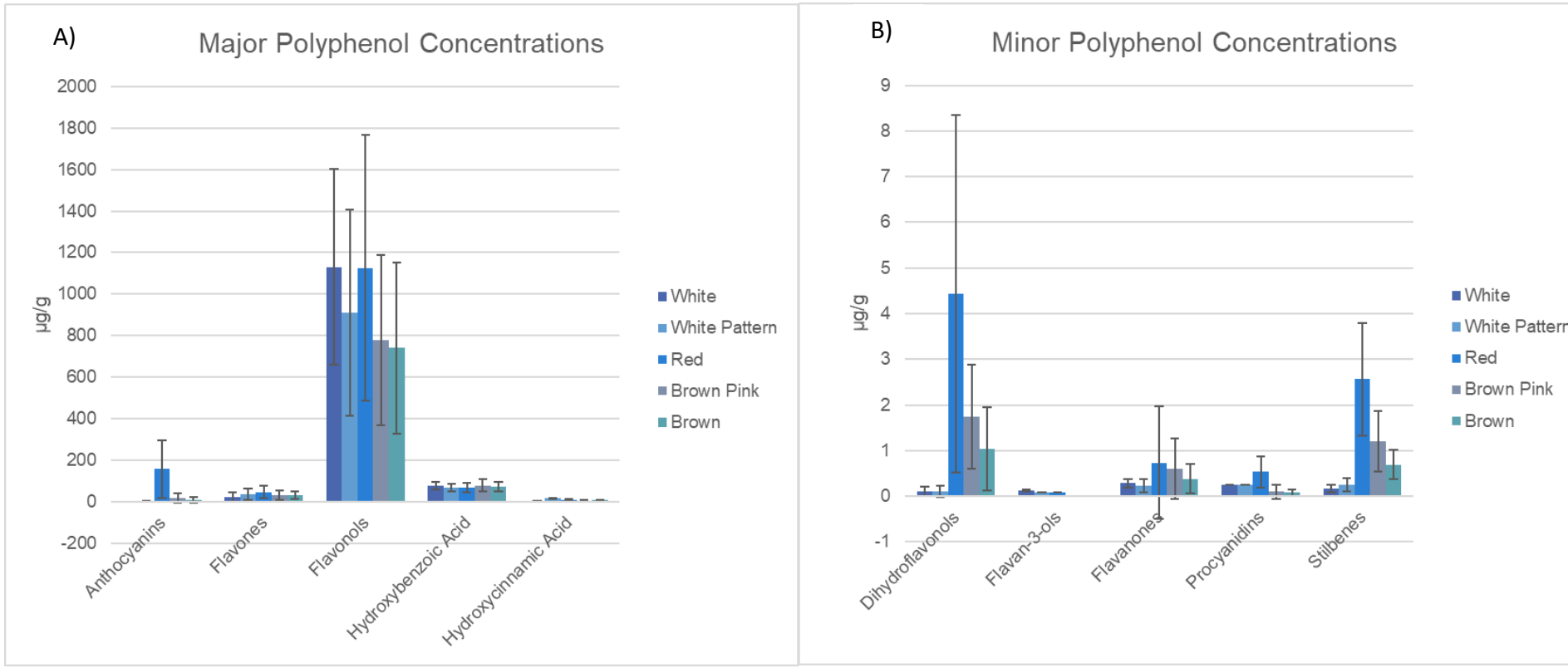


Figure D.1: Average polyphenolic class concentrations for each flower colour group in µg/g. A) Illustrates the major compound concentrations and B) illustrates the minor compound concentrations. Error bars account for the standard deviation within each group. N=4864 concentrations

D.2.1 Colour profile results

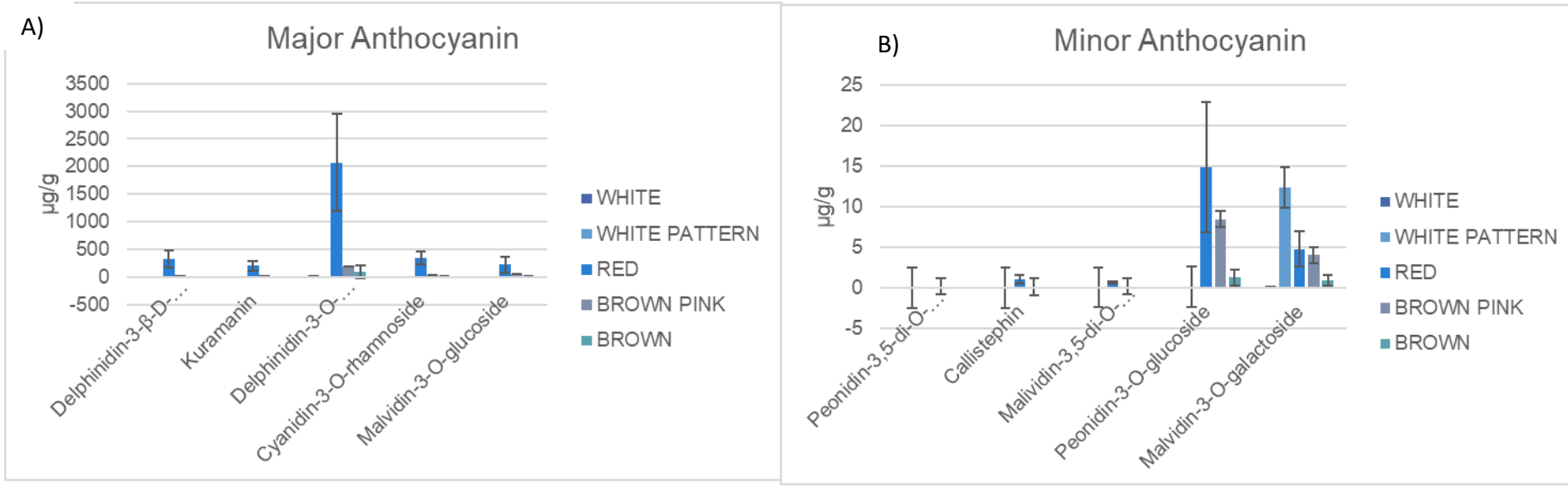


Figure D.2: Average anthocyanin concentrations for each flower colour group in µg/g. A) Illustrates the major compound concentrations and B) illustrates the minor compound concentrations. Error bars account for the standard deviation within each group. N=952 concentrations

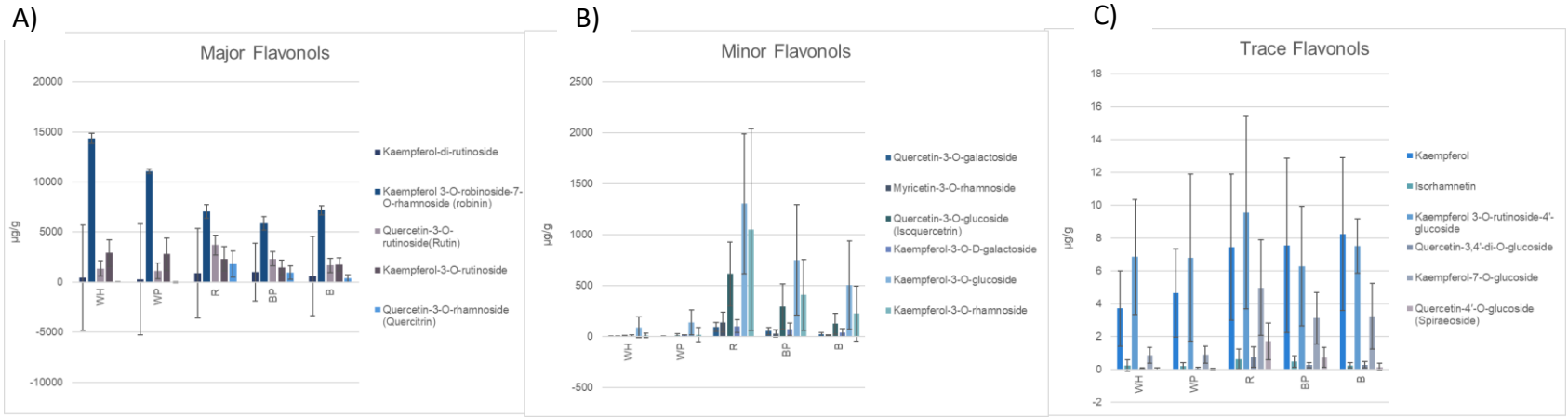
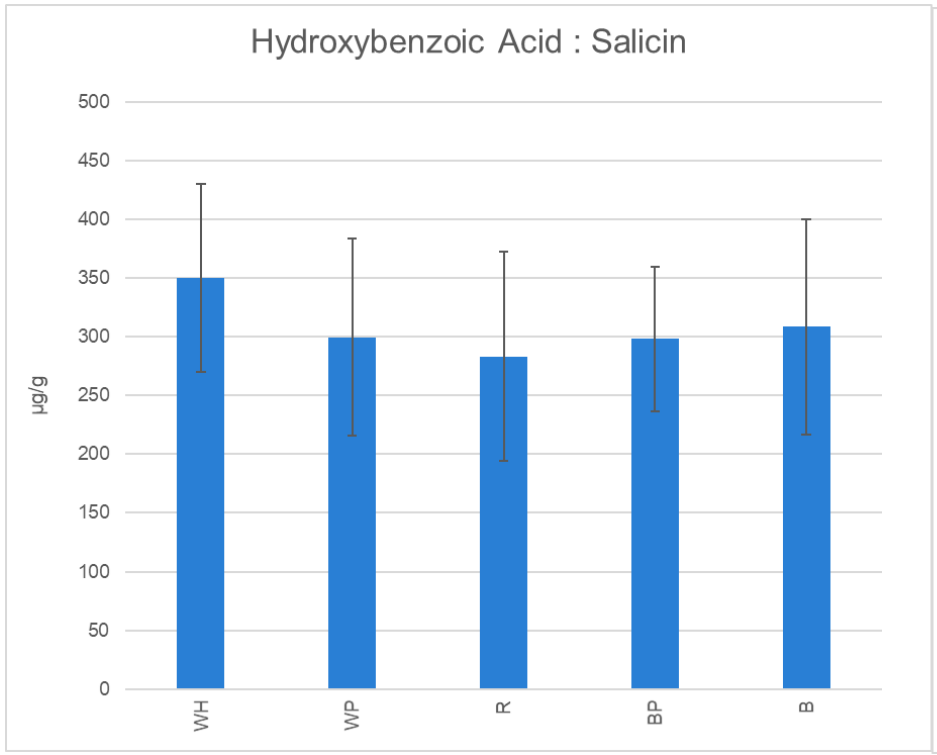


Figure D.3: Average flavonol concentrations for each flower colour group in µg/g. A) Illustrates the major compound concentrations, B) illustrates the minor compound concentrations and C) illustrates trace concentrations. Error bars account for the standard deviation within each group. N=1140 concentrations

A)



B)

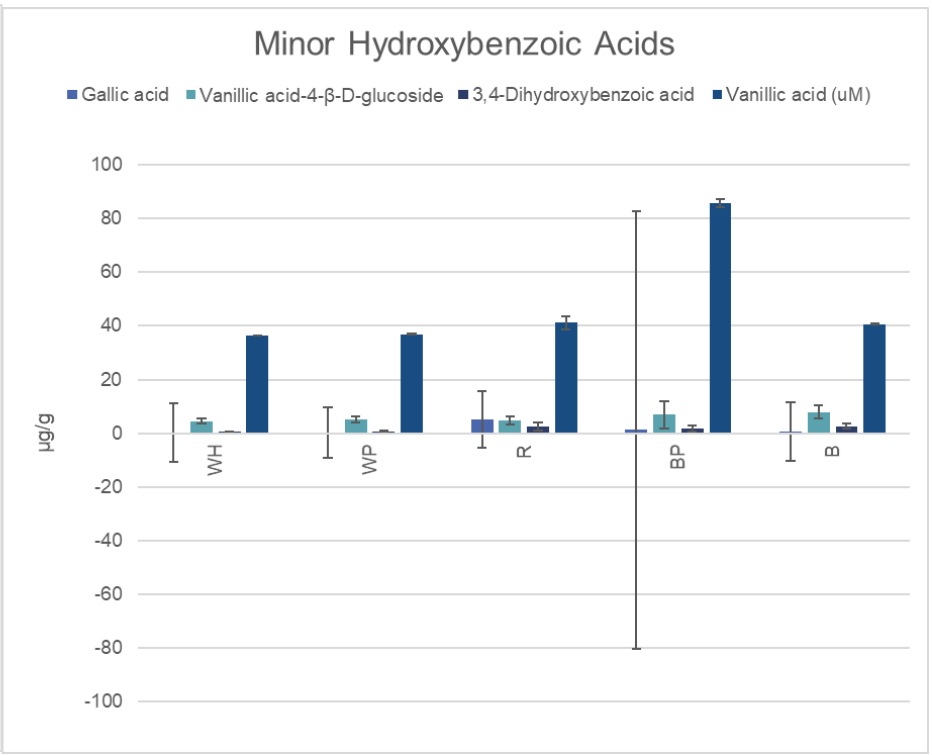


Figure D.4: Average hydroxybenzoic acid concentrations for each flower colour group in µg/g. A) Illustrates the concentration of Salicin and B) illustrates the minor compound concentrations. Error bars account for the standard deviation within each group. N=380 concentrations